

*PROTEIN TARGETS
FOR COVALENTLY BOUND
POLYCYCLIC AROMATIC HYDROCARBONS*

Cecilia Schelin



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by

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 PROTEIN TARGETS FOR COVALENTLY BOUND POLYCYCLIC AROMATIC HYDROCARBONS

Abstract
 The covalent binding of xenobiotics to rat liver microsomal proteins has been investigated. The binding was found to be highly specific and metabolic activation was required for binding. The binding patterns, examined with gel electrophoresis and fluorography, differed between different groups of xenobiotics and was influenced by pretreatment of the animals with phenobarbital and 3-methylcholanthrene. The highest amount of binding was seen with microsomes from 3-methylcholanthrene-pretreated rats.

Isolated benzo(a)pyrene metabolites had different binding patterns, but the composite pattern of the metabolites corresponded to the binding pattern of benzo(a)pyrene. All metabolites examined, including benzo(a)pyrene-4,5-epoxide, required metabolic activation before they were able to bind covalently to proteins. Benzo(a)pyrene-7,8-dihydrodiol bound most efficiently.

If the cytosolic fraction was included in the incubation mixture, covalent binding of benzo(a)pyrene to microsomal proteins decreased. Two cytosolic polypeptides bound benzo(a)pyrene covalently in the presence of microsomes and NADPH. They had the same mobility in the gel as the subunits of isolated glutathione transferase.

In reconstituted mixed-function oxidase systems, containing isolated liver enzymes, covalent binding of benzo(a)pyrene occurred to cytochrome P-450 isozymes and epoxide hydrolase. NADPH-cytochrome P-450 reductase was required for binding but did not bind the hydrocarbon itself.

An alternative method for quantitative determination of covalent binding to proteins was presented. The incubation mixture is applied on filter paper discs which are immersed in ethanol to precipitate the proteins and washed in organic solvents to remove unbound material before scintillation counting. The method is very rapid, simple and sensitive.

Key words
 rat liver, microsomal proteins, covalent binding, specific binding, polycyclic aromatic hydrocarbons, benzo(a)pyrene, cytochrome P-450

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LIST OF SEPARATE PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals.

- I Microsomal target proteins of metabolically activated aromatic hydrocarbons.
Anders Tunek, Cecilia Schelin and Bengt Jergil
Chem.-Biol. Interact. 27, 133-144 (1979)
- II Irreversible binding of isolated benzo(a)pyrene metabolites to specific rat liver microsomal proteins.
Cecilia Schelin, Anders Tunek, Bengt Jernström and Bengt Jergil
Mol. Pharmacol. 18, 529-535 (1980)
- III A rapid and sensitive method for determination of covalent binding of benzo(a)pyrene to proteins.
Håkan Wallin, Cecilia Schelin, Anders Tunek and Bengt Jergil
Chem.-Biol. Interact. 38, 109-118 (1981)
- IV Covalent binding of benzo(a)pyrene to rat liver cytosolic proteins and its effect on the binding to microsomal proteins.
Cecilia Schelin, Anders Tunek and Bengt Jergil
Biochem. Pharmacol. 32, 1501-1506 (1983)
- V Covalent binding of benzo(a)pyrene to cytochrome P-450_{βNF-B2} and other proteins in reconstituted mixed-function oxidase systems.
Cecilia Schelin, Håkan Wallin, James Halpert and Bengt Jergil
Submitted for publication

ABBREVIATIONS

AAF	2-Acetylaminofluorene
BaP	Benzo(a)pyrene
BaP-4,5-dihydrodiol	<u>trans</u> -4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene
BaP-7,8-dihydrodiol	<u>trans</u> -7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene
BaP-9,10-dihydrodiol	<u>trans</u> -9-10-dihydroxy-9,10-dihydrobenzo(a)pyrene
β NF	β -Naphthoflavone
DAB	N,N-dimethyl-4-aminoazobenzene
DMBA	7,12-Dimethylbenz(a)anthracene
GSH	Reduced glutathione
HPLC	High pressure liquid chromatography
MC	3-Methylcholanthrene
MFO	Mixed-function oxidase
PAH	Polycyclic aromatic hydrocarbon
PB	Phenobarbital
PCB	Polychlorinated biphenyl
UDP	Uridine diphosphate

1. INTRODUCTION

1.1. Historical background

Chemical carcinogenesis in humans was first documented by Hill in 1761, who found that excessive use of tobacco snuff caused nasal cancer (1). A few years later, in 1775, the surgeon Pott found a high incidence of scrotal cancer among men in London, who had been chimney sweeps in their childhood (2). Pott concluded that the close contact with coal tar and soot caused the cancer. Similar observations during the next century (3) stimulated attempts to induce tumors in experimental animals by application of particular chemicals. The first success was reported by Yamagiwa and Ichikawa in 1915 (4). They showed that coal tar applied on the ears of rabbits induced tumors at the site of application.

During the 1920's there was an intense search to identify the carcinogenic agent in tar. Several pieces of evidence were presented that the compound was a complex aromatic hydrocarbon (3). The first synthetic polycyclic aromatic hydrocarbon (PAH) to be identified as a potent carcinogen was dibenz(a,h)anthracene by Kennaway (5). A few years later, a carcinogenic PAH isolated from tar was identified as benzo(a)pyrene (BaP) by Cook, Hewett and Hieger (6).

Since that time an ever increasing list of compounds proven to be carcinogens in man has emerged. These compounds include industrial chemicals, drugs and naturally occurring chemicals (7,8). Epidemiologists have concluded that 80-90% of all human cancers are caused by environmental factors (9). Since definite data on the role of infectious viruses in the development of cancer is lacking (10), and since radiation plays a minor role (11), chemicals must be the predominant cause of cancer.

The first molecular target of chemical carcinogens was demonstrated by Miller and Miller in 1947 (12). They found that the hepatocarcinogen N,N-dimethyl-4-aminoazobenzene (DAB) would bind covalently to proteins in rat liver. Further investigations demonstrated a correlation between the extent of binding and the carcinogenicity of a number of compounds (13,14).

The emerging knowledge on the function of DNA and RNA in the storage and translation of genetic information suggested the importance of nucleic acid-carcinogen adducts in carcinogenesis. In the beginning of the 1960's evidence was provided that metabolically activated PAH would bind covalently to DNA as well as to proteins (15). A correlation between carcinogenicity and the extent of binding was found. Since then several investigations have been performed on the fate of administered carcinogens and their binding to macromolecules in target tissues. There are now strong indications that the covalent binding of carcinogens to macromolecules is a necessary, but not sufficient, step in the induction of tumors by chemical carcinogens (3). Correlations have also been found between covalent binding and cytotoxicity (16).

There has been an intense search to relate the structure of xenobiotics to their biological effects. It became obvious early that many carcinogenic and cytotoxic compounds had to be metabolized to chemically reactive products before they could exert their effects (7,15-19). The reactive metabolites formed are electrophilic in nature and will therefore readily react with nucleophilic groups in cellular macromolecules. It has been shown that this metabolic activation is carried out by a microsomal mixed function oxidase (MFO) system in the presence of NADPH and oxygen (17,18).

Gradually these and other studies have provided insights on the actions of chemicals in cells on a molecular level. However, the precise consequences of the covalent binding of any chemical in carcinogenic or cytotoxic processes are still unknown.

1.2. Enzymes involved in the metabolism of xenobiotics

Most xenobiotics are hydrophobic and would remain in the body if no metabolism, resulting in more water-soluble derivatives, took place. The responsible enzymes have a dual effect since they are both detoxifying and at the same time activate chemicals to carcinogenic, mutagenic and cytotoxic forms (7,19). The enzymes are present to some degree in all tissues examined, but they are particularly abundant in the liver. The enzymes are traditionally divided into two groups; phase I enzymes, which catalyze the introduction of one or more hydrophilic groups (such as

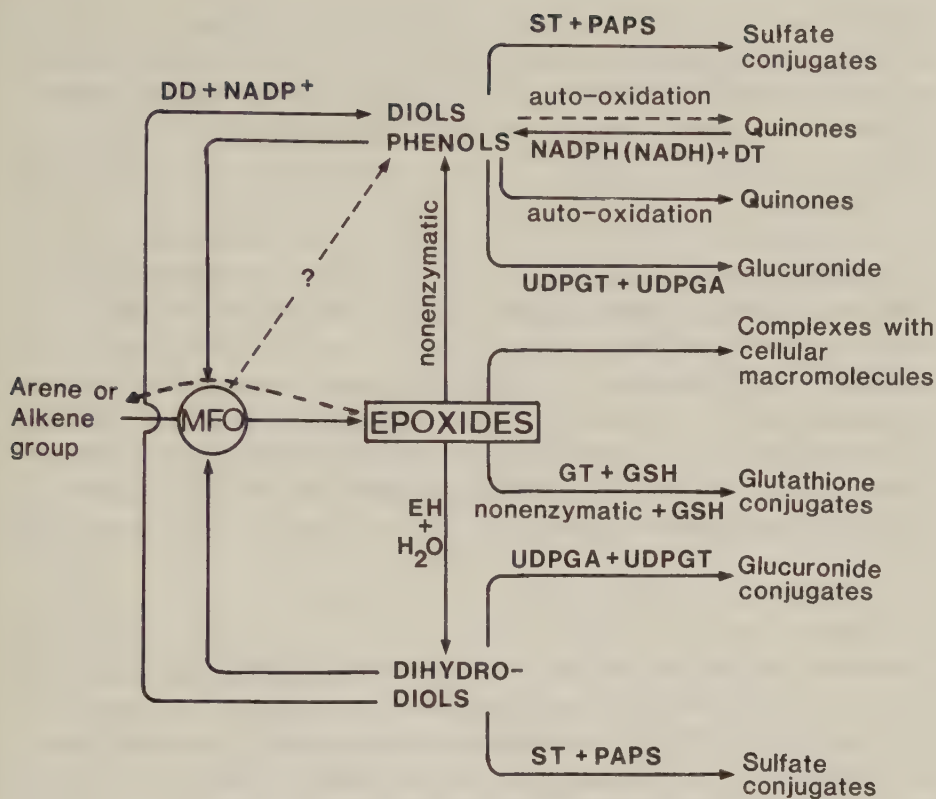


Figure 4. Pathway of metabolism of arene and alkene groups in xenobiotics

MFO	Mixed-Function Oxidase
EH	Epoxide Hydrolase
DD	Dihydrodiol Dehydrogenase
DT	DT-Diaphorase
ST	Sulfotransferase
GT	Glutathione Transferase
UDPGT	UDP-Glucuronosyl Transferase
PAPS	Adenosine-3'-phosphate-5'-phosphosulphate
GSH	Reduced Glutathione
UDPGA	UDP-Glucuronic Acid

hydroxyl groups) into the hydrophobic parent compound and phase II enzymes, which catalyze the conjugation of these metabolites to highly water-soluble compounds. The metabolites are mainly conjugated with GSH, UDPGA, or sulfate. The enzyme systems involved are presented in Fig. 1.

NADPH-cytochrome P-450 reductase

In the monooxygenation process, reducing equivalents are transferred from NADPH to the terminal oxidase, cytochrome P-450, via the flavoprotein NADPH-cytochrome P-450 reductase (20). Several pieces of evidence indicate that this reductase is identical with the flavoprotein known since 1950 as NADPH cytochrome c reductase (20,21). MFO systems reconstituted from isolated enzymes require the reductase for metabolic activity (22). The enzyme has been purified from rat liver microsomes and has been estimated molecular weight of 78 000 (23).

Cytochrome P-450

Cytochrome P-450 carries out the stereoselective insertion of one oxygen atom from O_2 into the substrate, while the second atom is used for the formation of water (20). The enzyme was found in 1958 by Klingenberg (24) and Garfinkel (25) who described a CO-binding pigment in liver microsomes. The pigment was named from the prominent peak at 450 nm observed in the reduced difference spectrum in the presence of CO. Omura and Sato showed that the pigment was a hemoprotein (26), and they performed the first solubilization and isolation studies (27).

The activity of cytochrome P-450, as well as other enzymes involved in the

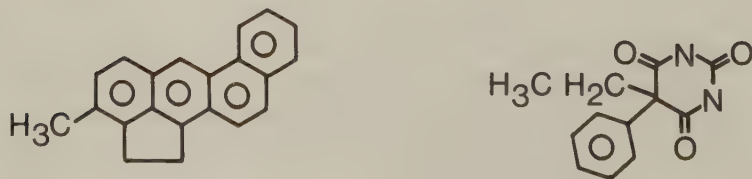


Figure 2. 3-Methylcholanthrene (MC) and phenobarbital (PB).

metabolism of xenobiotics, is markedly increased when animals are pretreated with various hormones, drugs, insecticides and carcinogens (28-31). This increase in activity is due to an induction of these enzymes. Barbiturates, for instance phenobarbital (PB), enhance the catalytic activity towards steroids, drugs and fatty acids (32-34). In contrast, PAHs such as 3-methylcholanthrene (MC) and β -naphthoflavone (β NF) cause an increase in the rate of oxidation of PAHs like BaP (35-37). The different kinds of inducers stimulate the synthesis of different isozymes of cytochrome P-450 (31,37,38). Several isozymes have been isolated, both constitutive and inducible forms. They exhibit differences in catalytic and immunological properties, optical and EPR characteristics, and responses to inhibitors, and their molecular weights vary between 47 000 - 57 000 (20,31,38,39). The inducibility of the isozymes is under genetic control (40,41) and varies between different tissues and animal species (19,42,43). It is also influenced by age and sex as well as nutritional and hormonal balance.

Cytochrome P-450 has a very broad substrate specificity, and mediates several metabolic reactions such as hydroxylations, oxidations, dealkylations, deaminations etc. (19). These activities have been reconstituted from isolated NADPH-cytochrome P-450 reductase, cytochrome P-450 and a heat-stable factor (22). This heat-stable factor could be replaced by phospholipids, and in particular phosphatidylcholine (44). In addition, cytochrome b₅ has been shown to increase the metabolic activity in a variety of systems (45,46). This role in cytochrome P-450-mediated reactions has been proposed to involve the transfer of a second electron in drug oxidations.

Epoxide hydrolase EC 3.3.2.3.

As early as in 1950 Boyland (47) proposed that intermediate arene oxides would be formed from aromatic hydrocarbons and suggested that they were important in carcinogenicity. This was later demonstrated by Jerina et al. (48). The chemically reactive oxides can be further metabolized by epoxide hydrolase to less reactive trans-dihydrodiols (49,50).

Epoxide hydrolase was first detected as a membrane-bound enzyme located mainly in the microsomal fraction (51). Apart from the principal form which metabolizes xenobiotics, a distinct microsomal form which metabolizes cholesterol-5,6-oxide has recently been described (52). Hammock and co-

workers (53) have also found enzyme activity in the cytosol using other analysis conditions. Epoxide hydrolase can be induced by several compounds, including PB (54), stilbenes (55) and antioxidants (56). Isolated epoxide hydrolase from rat liver contains a single peptide chain with a molecular weight of approximately 50 000 (50).

Glutathione transferase EC 2.5.1.18.

Glutathione transferase catalyzes conjugate formation between reduced glutathione (GSH) and various electrophilic compounds including arene and alkene oxides (57,58). The conjugates are further metabolized to mercapturic acids, which are finally excreted (59). Glutathione transferase has the additional role of binding a large number of hydrophobic compounds non-covalently, probably regulating the efflux of these compounds from the cell (60). Glutathione transferase may also bind reactive metabolites covalently, which leads to inactivation of the enzyme (60).

Several forms of glutathione transferase have been isolated from the cytosol of different tissues and species (58). Recently, Morgenstern et al. isolated and characterized a membrane-bound form from rat liver (61). Various compounds including PB and MC have been found to induce the enzymatic activity (62). In rat different cytosolic forms of glutathione transferase are dimers combined from four distinct subunits. The molecular weight of each subunit varies between 22 000 and 25 000 (63). Glutathione transferase has been calculated to represent 10 % of the total cytosolic protein in rat liver (64).

UDP-Glucuronosyl transferase EC 2.4.2.17.

Another conjugation enzyme is the membrane-bound UDP-glucuronosyl transferase. The enzyme makes lipophilic compounds more water-soluble by catalyzing the conjugation of glucuronic acid to hydroxyl, carboxyl, amino, and sulphhydryl groups (65,66). Administration of PB and MC induces this enzyme (67-70). The existence of multiple forms of UDP-glucuronosyl transferase has been suggested since the substrate specificity observed depends on previous treatment with various compounds.

Sulfotransferase EC 2.8.2.1.

Sulfotransferase catalyzes the transfer of the sulfate group from adenosine 3'-phosphate-5'-phosphosulfate (PAPS) mainly to aromatic and aliphatic hydroxyl groups (71-73). The resulting sulfuric acid esters are water-soluble and can be excreted readily. In addition dihydrodiols, epoxides and quinones have been shown to conjugate with the sulfate of PAPS (74). Treatment with MC increased the activity of sulfotransferase in rat (75). The enzyme has a molecular weight of approximately 65 000 and consists of two subunits in rat liver cytosol (73).

DT-diaphorase EC 1.6.99.2.

DT-diaphorase, or NAD(P)H-quinone oxidoreductase, is a flavoprotein which catalyzes the reduction of several dyes and quinones using NADH or NADPH as electron donors (76). The enzyme is inhibited by anticoagulants such as dicoumarol. Inducers of the microsomal MFO enzymes cause a several-fold increase in DT-diaphorase activity (77,78). The bulk of the hepatic enzyme is found in the cytosol, but mitochondria and microsomes also exhibit enzyme activity (79). DT-diaphorase is a dimer, and each subunit has a molecular weight of around 28 000.

Dihydrodiol dehydrogenase

In 1959 Ayengar et al. (80) described an enzyme which converted benzenedi-hydrodiol to catechol. This cytoplasmic NADP⁺-dependent dihydrodiol dehydrogenase is also capable of oxidizing dihydrodiol metabolites of PAHs (81). This metabolic route may contribute to the control of mutagenic metabolites, i.e. dihydrodiol epoxides (82). The protein is a monomer with a molecular weight of 35 000.

1.3. Benzo(a)pyrene metabolism

BaP has been used frequently as a model substrate in chemical carcinogenesis, since it is widely spread in the environment (83) and since it has mutagenic and carcinogenic properties after metabolic activation (84).

The metabolism starts with the introduction of an oxygen atom by the monooxygenase into one of the rings of the molecule, resulting in the formation of an electrophilic epoxide (30,47,85). The only epoxide that has been isolated is the relatively stable BaP-4,5-epoxide (86), but the identification of the hydrolysis products BaP-7,8-dihydrodiol and BaP-9,10-dihydrodiol (87) suggests an intermediate formation of at least two other primary epoxides. Evidence has also been presented for the formation of a BaP-2,3-epoxide which rearranges to 3-hydroxy-BaP (88). Such intermediate epoxides are probably the molecular species that will bind to macromolecules (19,30).

Epoxides can form conjugates with GSH either non-enzymatically or catalyzed by glutathione transferase (89-91). Alternatively, water addition can be catalyzed by epoxide hydrolase yielding trans-dihydrodiols (87,92). Four such dihydrodiols have been identified, namely 2,3-, 4,5-, 7,8-, and 9,10-dihydrodiol (91). The latter can be reduced by dihydrodiol dehydrogenase to the corresponding dihydroxy-BaP (93). Epoxides may also rearrange non-enzymatically to phenols via the so called NIH-shift (3,87,92). Six out of twelve possible phenolic isomers are known to be formed including 1-, 3-, 5-, 6-, 7-, and 9-hydroxy-BaP (91). 6-hydroxy-BaP is unstable, but can be predicted due to the presence of quinones (94). The 6-position does not sterically permit an epoxide intermediate, suggesting a more direct insertion of the oxygen atom (95). Finally, microsomes can reduce epoxides to their parent compounds (96,97).

Quinones are formed mainly by autooxidation of 6-hydroxy-BaP via the 6-phenoxy-radical (94), but also from further metabolism of 3-hydroxy-BaP (98) or lipid peroxidation of BaP (99). The identified quinones (1,6-, 3,6-, and 6,12-) can be reduced to diols by DT-diaphorase (100). Evidence has recently been presented that quinones may conjugate with GSH in a reaction catalyzed by glutathione transferase (99). BaP has also been shown to be metabolized to 6-hydroxymethyl-BP (101).

Figure 3. *An outline of Benzo(a)pyrene metabolism.*
For abbreviations, see Fig. 1.

Benzo(a)pyrene

Bay-region

MFO
NADPH
O₂

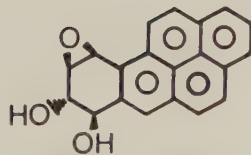
K-region

MACRO-
MOLECULAR
BINDINGGT+GSH
GSH

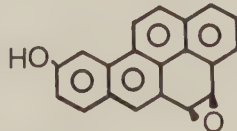
BP-7,8-epoxide

EH
H₂Oauto-
oxidation

BP-7,8-dihydrodiol

9-hydroxy-BP
(from BP-9,10-epoxide)BP-6,12-quinone
(from 6-hydroxy-BP)MFO
NADPH
O₂ST+PAPS
UDPGT+UDPGA
SULFATE AND
GLUCURONIDE
CONJUGATESMFO
NADPH
O₂

di-epoxide



9-hydroxy-BP-4,5-epoxide

MACRO-
MOLECULAR
BINDING

All identified phenols and dihydrodiols have been reported to conjugate with glucuronic acid and sulfate (74,102). Phenols are, however, better substrates for these reactions.

It has recently been found that phenols and dihydrodiols can undergo another round of metabolic activation catalyzed by the MFO (103,104). Secondary products of particular interest are the two stereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BaP, termed diolepoxide I and II (105,106). These intermediates can be hydrolyzed nonenzymatically to tetrols (105, 107), reduced by NADPH to triols (108), or conjugated with GSH (93). There is ample evidence today that diolepoxides are the metabolites of PAHs that most avidly will bind covalently to macromolecules (103,109-111), in addition to be the ultimate carcinogens (84).

The metabolic pattern of BaP can be changed by pretreatment with inducers, i.e. different forms of cytochrome P-450 will metabolize BaP in different positions. Thus, pretreatment with PB selectively increases metabolism in the 4,5-region, whereas MC increases metabolism in the 7,8,9,10-benzo-ring (31,104,107,111,112). Since different metabolites have different mutagenic and carcinogenic potencies (see below), pretreatment might have important biological effects.

2. COVALENT BINDING TO MACROMOLECULES

2.1. Covalent binding to nucleic acids

Several pieces of evidence exist that PAHs have to be metabolically activated before they exert their carcinogenic or cytotoxic effects. But the next step, the interaction with a critical subcellular target, is less clear. The most valid theory today, the somatic mutation theory, implicates DNA damage as the event initiating carcinogenesis (113,114). Covalent binding of reactive metabolites is one way to damage DNA and alter the genetic information. Such binding has been correlated with transformation, mutagenesis and carcinogenesis (19).

Several parameters have been investigated to try to correlate carcinogenicity with the chemical structure of various compounds. One attractive theory, presented by the Pullmans, was the K-region theory (7,115,116). It states that a PAH must possess an electron dense region, named the K-region, in order to be carcinogenic. This specific region corresponds to the 4,5-double bond of BaP. It was not until 1973 that Borgen et al. (109) found that liver microsomal metabolism of BaP-7,8-dihydrodiol resulted in a more extensive binding to added DNA than did metabolism of BaP and several other metabolites of BaP. Evidence was provided (103) that a 7,8-dihydrodiol-9,10-epoxide was the specific metabolite responsible for the binding. Levin et al. (84) have summarized their studies on the mutagenic and carcinogenic activities of several BaP derivatives and they conclude that the diolepoxide is the ultimate carcinogen derived from BaP.

These results, together with investigations performed with structural analogues of BaP, made Jerina and coworkers (117,118) postulate the "bay-region" theory. It is based on the unusual chemical reactivity associated with the benzylic position of a saturated angular benzo-ring, which forms part of a "bay-region" of a PAH (cf Fig. 2). Thus, the critical structural feature in BaP is an epoxide in the 9,10-position.

In systems containing microsomes from MC-pretreated rats and calf thymus DNA, Jernström (119) has shown that the main DNA-binding species is metabolically activated 9-hydroxy-BaP and, to a lower extent, BaP-7,8-dihydrodiol. The ultimate BaP metabolites responsible for binding were suggested to be 9-hydroxy-BaP-4,5-epoxide and BaP-7,8-dihydrodiol-9,10-epoxide in this and

other studies (120,121). Structural differences between the DNA-metabolite complexes formed were also seen. The diepoxide intercalates between base pairs, while the hydroxy compound is more exposed to the hydrophilic surrounding. These differences may partly explain why the diepoxide is more mutagenic and carcinogenic, while 9-hydroxy-BaP is comparatively harmless (119). Other metabolites of BaP such as epoxides, phenols, dihydrodiols, quinones and free radicals, have also been shown to bind to DNA (19).

BaP-metabolites are bound to nucleophilic sites in DNA, including such sites in deoxyguanosine, deoxycytidine, and also to phosphate groups (122-126). The exocyclic amino group of deoxyguanosine seems to be the site that is most susceptible to reactions with electrophilic metabolites of BaP (Fig. 4).

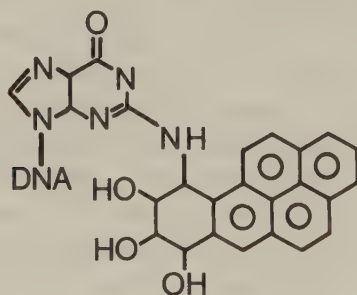


Figure 4. *The major adduct formed between BP-7,8-dihydrodiol-9,10-epoxide and nucleic acids.*

2.2. Covalent binding to proteins

An early clue to the possible molecular action of a carcinogen was the finding by the Millers in 1947 (12) of the covalent binding of metabolites of DAB to hepatic proteins in rats fed this dye. At that time several azodyes had been reported to be carcinogenic (13). The covalently bound DAB caused the liver proteins to turn pink in acid solution and light yellow in alkaline or neutral solvents (12). Like many other aminoazo dyes DAB is a sensitive acid-base indicator, and the colour change showed that the dye was associated with liver proteins. It was a chemically firm bond, since

the dye was not released by prolonged extraction with boiling organic solvents, by extraction with hot trichloroacetic acid, or by dialysis. The dye was released, however, upon destruction of the protein by trypsin or alkali. No binding was found to DNA with this rather insensitive method.

The Millers suggested that protein binding was important in the carcinogenic process induced by DAB (12,13). There was a correlation between the level of dye bound and the probability of tumor formation. The dye is a specific carcinogen for rat liver, and all binding was found in this tissue. No binding was seen in the liver of other animal species. Inclusion of riboflavin, cysteine, methionine and/or choline in the diet resulted in a decreased incidence of tumor formation. PAHs also reduced the level of protein-bound dye and its carcinogenicity.

Cytosolic proteins

Another observation was that no protein-bound DAB was found in tumors induced by this dye or its derivatives, even when these compounds were administered continuously (13). Further findings on the molecular level explained this intriguing observation. The major portion of the protein-bound dye in rat liver was present in the soluble protein fraction (127-129). On further fractionation by electrophoresis, Sorof et al. (130) found that 70 to 90 % of the bound dye was bound to slow moving-proteins which comprised 7 to 15 % of the total soluble proteins. Of considerable interest was the finding that the liver tumors contained greatly reduced amounts of this particular protein fraction (131). These so called "h"-proteins were resolved into six components by electrophoresis (132), where two sub-fractions, namely "h₃" and "slow-h₂", contained bound dye. The amount of the "slow-h₂" fraction increased when 2-acetylaminofluorene (AAF) or a carcinogenic aminoazo dye was administered, but not when the noncarcinogenic dye 2-methyl-DAB was given to rats. Investigations were performed in order to find out the enzymatic identity of this protein. Several enzyme activities were absent in hepatomas (133,134), but no enzyme could be identified as the "h"-protein.

These pioneering efforts, made particularly by the Millers (13), supported the "protein deletion" theory first suggested by Potter in 1944 (135). The facts presented above supported the hypothesis that carcinogenic dyes induce neoplastic changes through the gradual deletion of key proteins essential

TABLE I

A survey of available data on the covalent binding of xenobiotics to cellular proteins

Compound	Species	Fraction ¹	Reference	No.
Acetaldehyde	Rat	Microsomal	Nomura et al.	149
2-Acetylaminofluorene	Rat	Soluble	Miller et al.	138
			Weisburger et al.	139
	Rat	Nuclear	Barry et al.	150
	Mouse	Microsomal	Potter et al.	151
	Human	Microsomal	Dybing et al.	152
	Rat	Cytosolic	Blackburn et al.	153
Acrylonitrile	Rat	Microsomal	Peter et al.	154
Aflatoxins	Rat	Soluble (in L,K,S and SI)	Lijinsky et al.	155
	Rat	Microsomal	Gurtoo et al.	156
	Rat	Nuclear	Vaught et al.	157
2-Aminofluorene	Rat	Microsomal	Hultin	158
Amphetamine	Rabbit	Microsomal	Uehleke	159
Aniline	Rat	Microsomal	Hultin	158
Antipyrine	Rabbit	Microsomal	Uehleke	159
Benzene	Rat	Microsomal	Tunek et al.	160
Benzo(a)pyrene	Mouse	Skin proteins	Miller	136
	Mouse	Soluble/Partic.	Heidelberg et al.	14
	Rabbit	Microsomal	Uehleke	159
	Rat	Microsomal	Tunek et al.	1
	Rat	Nuclear	Vaught et al.	161
			Pezzuto et al.	162
	Rat	Cytosolic	Reeve et al.	163
			Schelin et al.	164
Bromobenzene	Rat	Micros./Cytos.	Brodie et al.	165
Butylated hydroxy-toluene	Rat	Microsomal	Nakagawa et al.	166
CBrCl ₃	Rat	Microsomal	Sipes et al.	167
CCl ₄	Rat	Microsomal	Sipes et al.	167
	Rabbit	Microsomal	Uehleke et al.	168
	Rat/Mouse	Nuclear	Diaz-Gomez et al.	169
CHCl ₃	Rabbit	Microsomal	Uehleke	159
	Rat/Mouse	Nuclear	Diaz-Gomez et al.	170

Compound	Species	Fraction ¹	Reference	No.
Chloramphenicol	Rat	Microsomal	Pohl et al.	171
Chlorobenzene	Rabbit	Microsomal	Uehleke	159
	Rat	Microsomal	Selander et al.	172
Chlorpromazine	Rabbit	Microsomal	Uehleke	159
Cyclophosphamide	Rat	Microsomal	Gurtoo et al.	173
Cytembena	Rat	Microsomal	Mayo et al.	174
2,4-Diaminoanisole	Rat	Microsomal	Dybing et al.	175
1,2,5,6-Dibenzanthracene	Mouse	Skin Nuclear/ Soluble/Partic.	Wiest et al.	137
	Mouse	Skin Cytosolic	Tasserone et al.	144
Diethylstilbestrol	Rat	Microsomal	Krishna et al.	176
N,N-Dimethyl-4-aminoazobenzene (and related azo dyes)	Rat	Homogenate	Miller et al.	12
	Rat	Soluble	Price et al.	128
	Rat	Microsomal	Hultin	18
	Rat	Cytosolic	Ketterer et al.	142
	Rat	Nuclear	Bakay et al.	177
7,10-Dimethylbenzanthracene (and other anthracene derivatives)	Mouse	Skin Soluble/ Particulate	Heidelberger et al.	14
	Rat	Nuclear	Jungmann et al.	178
	Rat	Adrenal gland Micros./Cytos.	Montelius et al.	179
Diphenylhydrantion	Rabbit	Microsomal	Uehleke	159
Estradiol	Rat	Homogenate	Riegel et al.	180
Estrone	Rat	Homogenate	Szego	181
	Rat	Microsomal	Marks et al.	182
Ethane 1,2-dichloro 1,1,2,2-tetrachloro	Rat/Mouse	Lung Microsomal	Banerjee et al.	183
	Rat	Microsomal	Halpert	184
Ethinylestradiol	Rat	Microsomal	Kappus et al.	185
	Rat	Cytosolic	Maggs et al.	186
Ethionine	Rat	Soluble	Sorof et al.	187
	Rat	Micros./Cytos./ Nuclear (in L, K,P and S)	Farber et al.	188
Ethylene 1,2,2-trichloro	Rat/Mouse	Microsomal	Banerjee et al.	189
	Rat/Mouse/ Human	Micros./Hepatocytes (in L and lung)	Miller et al.	190
dibromide and dichloride	Rat/Mouse	Microsomal (in L and ST)	Banerjee et al.	191

Compound	Species	Fraction ¹	Reference	No.
Furosemide	Mouse	Microsomal	Potter et al.	192
Halothane	Rabbit	Microsomal	Uehleke et al.	193
	Rat	Microsomal	de Groot et al.	194
Hexachlorophene	Rabbit	Microsomal	Uehleke	159
Imipramine	Rabbit	Microsomal	Uehleke	159
Isoniazid	Mouse	Microsomal	Thorgeirsson et al.	195
Methylcholanthrene	Mouse	Skin Soluble/ Particulate	Heidelberger et al.	14
	Rat	Cytosolic	Litwack et al.	143
	Mouse	Cytosolic	Tasserone et al.	144
	Rat	Microsomal	Tunek et al.	I
Naphthalene	Rat	Microsomal	Hesse et al.	196
2-Naphtylamine	Rat	Microsomal	Van der Decken et al.	197
6-Nitrobenzo(a)pyrene	Rat	Proteins (in L,K,LU and S)	Martin et al.	198
Nitrosamines	Rat	Tissue slices	Magee et al.	199
	Rat	Nuclear	Tuberville et al.	200
	Human	Erythrocytes	Kim et al.	201
Paracetamol	Rat	Microsomal	Potter et al.	151
	Mouse	Cytosolic	Wendel et al.	202
Parathion/Malathion	Mouse	Microsomal	Stevens	203
PCBs	Rat	Microsomal	Shimada et al.	204
Chlorobiphenyl	Rat	Microsomal	Wyndham et al.	205
Dichlorobiphenyl	Rat	Microsomal	Hesse et al.	206
Tetrachlorobiphenyl	Monkey	Microsomal	Seymour et al.	207
Phenobarbital	Rabbit	Microsomal	Uehleke	159
Phenol	Rat	Microsomal	Tunek et al.	I
Pyribenzamine	Rat	Microsomal	Rao et al.	208
<u>trans</u> -Stilbene	Rat	Microsomal	Docks et al.	209
<u>trans</u> -/ <u>cis</u> -stilbene- oxide	Rat	Microsomal	Schelin et al.	210
Thioacetamide	Rat	Micr./Cyt./Mit.	Dyroff et al.	211
Vinylchloride/bromide	Rat	Microsomal	Guengerich	212
Urethan	Rat	Cytosol/Nuclear (in L,K,LU,S and Skin)	Prodi et al.	213

for the control of growth.

Evidence for similar carcinogen-protein complexes was also found for compounds as BaP (136), MC (14), dibenzanthracene (137), AAF (138,139), and several anthracene derivatives (14) in different animal species. Usually extensive protein binding was reported for carcinogenic compounds, whereas little binding was found for noncarcinogenic ones (13,134). Other researchers, however, did not find a relation between protein binding and carcinogenic activity (140).

In mice carcinogenic hydrocarbons bound principally to two protein fractions separated by starch gel electrophoresis (141). The extent of binding to one of them, protein fraction I, was directly proportional to the carcinogenic activity of the PAH. The protein had the same mobility in the gel as the "slow-h₂" protein of rat liver, and it was also absent in tumors.

The cytosolic binding proteins were purified independently by several research groups (142-145) and compared. Litwack et al. (146) presented evidence in 1971 that one "h" protein and protein fraction I were identical, and named this protein ligandin. This abundant protein could also bind several other substances noncovalently, including heme, bilirubin and steroids (146,147). A couple of years later it was shown that ligandin was identical to one form of glutathione transferase, namely glutathione transferase B (147). Today, evidence exists that several cytosolic proteins may bind various compounds covalently or noncovalently (148). Covalently bound compounds have been summarized in Table I.

Microsomal proteins

In 1957 it was demonstrated that DAB could bind covalently to liver endoplasmic reticulum (18), a structure already known for its drug oxidizing capacity. Hultin and coworkers also showed irreversible binding of aniline

¹If not stated otherwise, subcellular fractions from liver have been examined. The abbreviations used are: L, liver; LU, lung; K, kidney; P, pancreas; S, spleen; SI, small intestine and ST, stomach.

and 2-aminofluorene (158) as well as 2-naphtylamine (197) to rat liver microsomal proteins during aerobic incubations in the presence of NADPH.

As shown in Table I, many compounds are now known to bind covalently to microsomal proteins. The compounds examined more closely often show the same behaviour: requirement of metabolic activation, increased binding after pretreatment of the animals with inducers, and a decreased binding in the presence of different MFO inhibitors, GSH or phase II enzymes. However, towards the end of the 1970's there was still no information available concerning the microsomal target proteins for these compounds.

Nuclear proteins

The nuclear proteins can be divided into histones and nonhistone proteins (214). In general, carcinogens tend to bind more to nonhistone proteins than to histones, and more to histones than to DNA, but agreement between different research groups as to the quantitative level is poor (148). Since the histones are well defined and easily separated, several investigations have been concerned with the binding specificity of carcinogens to these proteins.

Bakay et al. (177) performed one of the first systematic studies on the influence of azo dyes on nuclear proteins. The nuclear sap proteins contained 86 % of the bound dye present in the nuclei, and of this 50 % had electrophoretic properties similar to those of the "h₂-5S" protein of the cytoplasm.

Jungmann and Schweppe (178) found covalent binding of 7,12-dimethylbenzo(a)-anthracene (DMBA) to histones, preferentially, histones H1 and H2B and non-histones proteins in rat liver. BaP is another PAH that will bind covalently to nuclear proteins (161,162). In hamster embryo cells the nucleosomal core histones H2A and H3 bind BaP-7,8-diol-9,10-epoxide (215). The same research group has presented evidence that the reverse diol-epoxide, BaP-9,10-diol-7,8-epoxide, labels a nuclear protein with a molecular weight of 32 000 (216). This protein is not identical to histone H1, which has been suggested to be a target by others (217), using a different experimental system. In mouse embryo cells, BaP bound to histones H2B and H3 as well as to a protein which migrates close to H1 in SDS gel electrophoresis (218). In rat liver, most incorporation was found to nonhistone proteins, but also to histones as H1 (219). Other PAHs that have been shown to bind to nuclear proteins

are MC and benz(a)anthracene in hamster embryo cells, where they will bind mainly to histone H2A and H3 (220).

The reactive carcinogen formed from AAF is N-hydroxy-AAF. Esters of this compound, as sulfate esters, are the ultimate carcinogens that react with nucleophiles in the cell (3,214,221). Activated AAF has been shown to bind to histones H3 and H4 (150), H2A and H4 (178), and to nonhistone proteins (222) in rat liver. Nitrosamines, powerful alkylating agents, will also bind to histones (200,223). Other compounds that have been shown to bind to nuclear proteins are aflatoxins (157), CCl_4 (169), CHCl_3 (170) ethionine (191), thioacetamide (211) and urethan (213).

3. PRESENT STUDY

3.1. Aims

When BaP was injected intraperitoneally into rats, it appeared rapidly in the liver (224). Ten per cent of this BaP became bound to tissue macromolecules, of which a predominant part was proteins. Binding occurred most rapidly to the microsomal fraction. The covalent binding of xenobiotics to proteins has been suggested to be involved in cytotoxic (16,19) and carcinogenic (163,214,225) processes. Our starting point was a conviction that the massive binding to proteins has physiological significance.

Several investigations have been concerned with the identity of the ultimate BaP metabolite that would bind to DNA, and with the binding site in DNA. Evidence and theories have been presented on how covalent binding of xenobiotics may affect DNA causing transformation, mutation, and cancer. When this work started, several proteins had been identified as targets in the nuclear and cytosolic fractions, but there was little information available concerning the microsomal protein targets. In the present investigations our main purpose has been to provide information about microsomal target proteins in order to examine possible biological effects. We have tried to answer the following questions. Is the binding of xenobiotics to microsomal proteins specific or does it occur randomly? If the binding is specific, what is the identity of the target proteins? Which metabolite(s) will bind and to which part(s) in the protein molecules will they bind?

3.2. Specific binding to microsomal proteins (I)

The first investigation was concerned with the specificity of covalent binding of various hydrocarbons to microsomal proteins. Simultaneously, the requirement of metabolic activation, influence of inducers and effects of GSH, UDPGA and substrate concentration were examined. Radiolabelled hydrocarbons (BaP, MC, benzene, chlorobenzene and phenol) were incubated with rat liver microsomes and NADPH. The specific metabolite binding patterns were examined by SDS-polyacrylamide gel electrophoresis followed by fluorography. This technique was a new way of determining the specific binding of xenobiotics to microsomal proteins. Labelled protein bands, using either ^3H - or ^{14}C -labelled compounds, could easily be compared with

the protein pattern.

We showed that covalent binding occurred to a few proteins in the microsomes. Two kinds of binding patterns were observed, one for benzene, chlorobenzene and phenol, and another one for BaP and MC. The first pattern showed a dominant radiolabelled band at M_r 72 000 which was strong in both control and pretreated animals. Additional bands in the 50 000 - 60 000 M_r region were seen particularly in MC-pretreated microsomes. BaP and MC, on the other hand, bound to several components in the 50 000 - 60 000 M_r region and to two components of M_r 68 000 and 72 000. These binding patterns were highly influenced by pretreatment of the animals with PB or MC. Thus, BaP preferentially reacted with a protein of M_r 60 000 in microsomes from PB-treated rats while microsomes from MC-treated rat showed a new protein band at M_r 56 000 which bound BaP. Quantitatively the highest amount of binding was seen with microsomes from MC-pretreated rats. An NADPH-generating system was required for binding, indicating that metabolic activation preceded binding. GSH and UDPGA greatly reduced the binding.

This was the first investigation performed on the binding specificity of xenobiotics to microsomal proteins. We suggested that the differences in metabolite binding patterns reflect differences in the routes of metabolite formation and that activated hydrocarbons are likely to bind to proteins close to their site of formation. The latter suggestion implies that an enzyme that catalyses the formation of the metabolite would itself receive most of the binding provided it contains an exposed reactive group. This was supported by the radioactive band of M_r 56 000 which was obtained after incubation of all five hydrocarbons with microsomes from MC-pretreated rats. This protein had the same mobility in the gel as the major form of cytochrome P-450 from MC-pretreated rats. The nonrandom distribution of BaP bound to microsomal proteins stimulated further studies to identify target proteins and investigations concerning the effect of such covalent binding on the function of the target proteins.

Later studies by other research groups have shown that other carcinogens as well will bind specifically to microsomal proteins. Thus, Kaderbhai et al. (226) showed that AAF bound specifically to proteins in rat liver microsomes. The labelling pattern was strongly influenced by pretreatment of the animals with inducers of drug-metabolizing enzymes. They suggested

that isozymes of cytochrome P-450, cytochrome b_5 and their reductases were targets. Epoxide hydrolase had been reported earlier to bind AAF covalently (227). Montelius and coworkers (179) investigated the covalent binding of DMBA to microsomal and cytosolic proteins in rat adrenal gland. The binding occurred to a few specific proteins and they proposed that cytochrome P-450 and glutathione transferase were targets. The covalent binding of carbon tetrachloride to microsomal proteins is also highly specific (228). CCl_4 is activated to trichloromethyl radicals, mainly by the cytochrome P-450 form induced by PB-pretreatment. The investigation suggested also that this isozyme was a target for CCl_4 . In studies using polychlorinated biphenyls (PCBs), a non-random distribution of protein-bound compounds was found (229). Cis- and trans-stilbene oxide also bind specifically to microsomal proteins (210).

3.3. Covalent binding of BaP-metabolites (II)

Several investigations have been concerned with the metabolite(s) that become bound covalently to DNA. Different metabolites have been shown to bind to different extents and to have different mutagenic and carcinogenic effects (19,31,118). No information was available, however, on the binding of different BaP-metabolites to microsomal proteins. The purpose in paper II was to investigate if BaP metabolites became bound to different extent and if they bound to different target proteins. One interesting question was whether the BaP metabolite suggested to be the ultimate carcinogen also was the ultimate binding species to proteins?

Seven radiolabelled BaP-metabolites were isolated by HPLC and incubated with microsomes from control and pretreated rats. All metabolites examined, including the BaP-4,5-epoxide, required metabolic activation before they were able to bind covalently to proteins. BaP-7,8-dihydrodiol bound most efficiently. All metabolites, except 3-hydroxy-BaP, bound more extensively to microsomes from MC-pretreated rats than to microsomes from PB-pretreated ones.

The metabolite binding patterns, examined by electrophoresis and fluorography, were different for different metabolites. The most extensive binding occurred to a few proteins in the 50 000 - 60 000 M_r region. In microsomes from PB-pretreated animals three proteins became labelled, and the K-region metabolites bound exclusively to one of them (M_r 60 000). Using MC-induced

microsomes, the BaP metabolites bound to several proteins of which one comigrated with the major form of cytochrome P-450 from MC-treated animals. Surprisingly, BaP-7,8-dihydrodiol did not seem to bind to this component. The composite pattern of the metabolites corresponded to the binding pattern obtained with BaP. We concluded that the binding species were likely to be secondary metabolites, for instance BaP-7,8-dihydrodiol-9,10-epoxide, in analogy with the activation preceding binding to DNA.

3.4. An alternative method for quantitative determination of covalent binding (III)

A limitation when analyzing the quantitative covalent binding of xenobiotics to proteins was the lack of a convenient method. In the established methods excess radiolabelled compounds were removed after incubation by extraction with organic solvents and/or precipitation of the proteins followed by repeated washings of the precipitate (160-162,178,207,230). These methods were time-consuming and required large amounts of material. This is a serious problem when the binding of metabolites to isolated enzymes is to be studied and when radiolabelled substrates of high specific activities are used. Another method, based on dialysis in the presence of SDS to remove excess material (231), measures binding to macromolecules in general, i.e. including nucleic acids and complex lipids. Finally, one method using precipitation of the proteins on filter paper discs existed (168). Trichloroacetic acid was used for precipitation and washing of the filters, a technique not suitable for analysis of PAHs.

This paper presents an alternative analytical method for quantitation of BaP bound covalently to proteins. After incubation of radiolabelled BaP with microsomes and NADPH, the mixture is applied to filter paper discs. These are immersed in ethanol to precipitate the proteins. Excess BaP is then extracted with organic solvents, while the proteins are still precipitated on the discs. Finally, the discs are dried and counted in a liquid scintillation counter. This work also includes an investigation of the incubation conditions required for optimum binding of BaP to microsomal proteins. The maximum binding observed after nearly 4 hours, around 20 nmol bound per mg protein, corresponds to a unity molar ratio between incorporated BaP and microsomal proteins, assuming an average molecular weight of 50 000 for the proteins. The use of NADPH instead of an NADPH-generating system gave higher levels of metabolite binding.

Compared to other methods this method offers several advantages: it is very rapid, simple and sensitive, the background is usually very low, there is a quantitative recovery of precipitated proteins, and the incorporation can be measured accurately using a small amount of protein. The method can be adopted easily for the analysis of other covalently bound xenobiotics, as DMBA (179), trichloroethylene (190), cis- and trans-stilbene oxide (210), and phenol (232) using microsomes or other subcellular fractions (IV,V).

3.5. Influence of cytosolic proteins (IV)

It is of great importance to evaluate if in vitro experiments are comparable with the situation in vivo. One way to approximate the situation in the cell, might be to include the cytosolic fraction in the incubation experiments. This fraction was, therefore, included to find out whether it influenced the binding of BaP to microsomal proteins and if any cytosolic proteins would bind BaP covalently.

The cytosolic proteins themselves did not bind BaP covalently when incubated with NADPH, but binding to cytosolic proteins was seen if microsomes were included in the incubation mixture. This indicates that metabolic activation by microsomal proteins is a requirement for binding to cytosolic proteins. The binding to cytosolic proteins was most extensive when microsomes from MC pretreated rats were used. The binding to microsomal proteins decreased when incubations were performed with increasing concentrations of cytosol. Two cytosolic polypeptides were the targets for BaP. They had the same mobility in the gels as the subunits of purified glutathione transferase B. These subunits also reacted covalently with BaP when isolated transferase B was incubated with microsomes and NADPH.

Our results showed that the two soluble components that became modified covalently by BaP most probably were the subunits of glutathione transferase B (ligandin). This is in agreement with recent findings (163, 164). Other compounds have also been shown to bind covalently to ligandin (60,146, 202). In some of these cases the catalytic function of the transferase was reduced (60,163). Thus, these carcinogens effectively inhibited one major route of their own detoxification. Another interesting observation was the decrease in binding to microsomal proteins in the presence of cytosol. It would be interesting to examine whether this competition

between microsomal and cytosolic proteins for BaP metabolites has any physiological significance. Glutathione transferase has been shown earlier to reduce the incorporation of the ultimate carcinogen of BaP (233) and aflatoxin B₁ (234) to DNA.

3.6. Covalent binding to isolated MFO enzymes (V)

This investigation was concerned with the identification of microsomal target proteins for BaP metabolites. Indirect evidence from our earlier studies indicated that BaP became bound covalently to the MC-induced form of cytochrome P-450. Radiolabelled protein bands around M_r 50 000 and 72 000 suggested that other cytochrome P-450 forms, epoxide hydrolase, and NADPH-cytochrome P-450 reductase could be targets. If this were the case, it would be consistent with the theory that metabolites will bind to proteins close to their site of activation.

In order to identify microsomal target proteins, we incubated BaP with reconstituted MFO systems. These systems were mixtures of different isolated liver enzymes, namely NADPH-cytochrome P-450 reductase, the two major forms of cytochrome P-450 from PB and β NF pretreated rats, and epoxide hydrolase. The β NF-induced isozyme of cytochrome P-450 bound BaP covalently in the presence of NADPH. The reductase was required for binding, and a maximum rate of adduct formation was obtained at 8 Units of reductase per nmol cytochrome P-450. BaP was bound to the cytochrome, but not to the reductase, as shown by gel electrophoresis and fluorography. Approximately 6 molecules of BaP became bound to each cytochrome P-450 molecule during prolonged incubations. No binding occurred in systems containing the PB-induced isozyme, but both isozymes incorporated BaP to about the same extent when they were incubated together in the presence of reductase and NADPH. Metabolically activated BaP also bound covalently to purified epoxide hydrolase in reconstituted systems containing reductase, β NF-induced cytochrome P-450, and NADPH.

This was the first study to show conclusively that BaP will bind covalently to the β NF-induced form of cytochrome P-450. Other xenobiotics have also been shown to bind covalently to cytochrome P-450 isozymes in reconstituted systems, including BaP-7,8-dihydrodiol (235), chloramphenicol (236), and parathion (237). The covalent modification by the two latter compounds was followed by a decrease in the activity of PB-induced cytochrome P-450.

Such a suicide inactivation of cytochrome P-450 has also been found with CCl_4 (238,239), halothane (194), and allylisopropylacetamide (240) using microsomes or in vivo conditions. The inactivation in these cases is probably due to a covalent binding to the heme moiety, followed by a loss of cytochrome P-450.

4. GENERAL DISCUSSION

4.1. Binding specificity

Since the hydrocarbon metabolites appeared to bind specifically rather than randomly to microsomal proteins and since different protein binding patterns were observed for different hydrocarbons, the question arose which factors would determine the binding specificity. One possibility suggested in paper I is that reactive metabolites will bind to any macromolecule with an exposed group. This implies that any newly formed reactive metabolite most likely will bind to a reactive protein close to the site of metabolite formation. The enzyme that catalyzes the formation would itself receive most of the binding provided it has an exposed reactive group. We found later (V) that cytochrome P-450 was a target for reactive metabolites, as was epoxide hydrolase (V, 227) which is found in close connection with cytochrome P-450 in the microsomes (241). On the other hand, NADPH-cytochrome P-450 reductase did not bind activated BaP, although the reductase also interacts with cytochrome P-450 (242). An explanation to this can be that the reductase lacks exposed reactive groups. An alternative possibility is that the kind of metabolite formed (for instance a semiquinone or an epoxide) determines the target specificity. In paper II, evidence was presented that different metabolites indeed have different binding specificities.

Although both these possibilities seem to be valid, other factors may also determine the binding specificity. Thus, the binding of xenobiotics to specific proteins may reflect a biological function. One example is glutathione transferase B (60,163) which in addition to its catalytic activity perhaps has the, so far speculative, function of protecting other more sensitive components of the cell from covalent attack by binding reactive metabolites itself (64). Other proteins may also have a similar function. Another biological function of several proteins which, however, usually involves a noncovalent binding, is to transport various compounds. Reactive sites in proteins, metabolites that will bind, and possible physiological significance of the covalent binding will be discussed below.

4.2. Nucleophilic reactive sites

Proteins have several nucleophilic groups, including methionine, cysteine, histidine, tyrosine, and tryptophan residues (91,214,243), which may react with activated carcinogens.

Numerous investigations have dealt with the covalent binding to sulfur groups. Enzymes with SH groups crucial for their activity were early shown to be sensitive to carcinogens (133,134,138,244). The carcinogens were suggested to react with the SH groups causing enzyme inactivation. Such reactions were proposed to initiate carcinogenesis. The first more direct evidence that cysteine residues were actually involved in the binding of PAH was provided by Corbett and Nettesheim (245). They treated protein fractions in lung and skin, to which DMBA and MC had bound, with Raney nickel. The radioactive hydrocarbon derivatives released suggested that the covalent binding to protein involved a sulfur atom, probably from cysteine. Cysteine residues have also been found to bind azo dyes (246), AAF (221), chloramphenicol (246), cyclophosphamide (173), nitrosamines (248), and parathion (237).

Administration of AAF and DAB to animals also yielded methionine, tyrosine, and tryptophan adducts (221,214,246). Nitrosamines alkylate methionine and histidine residues, in addition to cysteine (248). Recently, lysine has been shown to be covalently modified by xenobiotics. Adduct formation with this amino acid residue has been reported for chloramphenicol (249), BaP (217), and thioacetamide (211). In an investigation with 14 different polyamino acids in vitro, several carcinogens reacted preferentially with poly-cysteine (250). Reactions were also noted with polyhistidine, polylysine, polymethionine, polyarginine, and in some instances, polyserine. Gurtoo et al. (173) suggested that cyclophosphamide would bind to amino groups in proteins via Schiff's base formation. Finally, metabolites of CCl_4 , such as the $\cdot\text{CCl}_3$ radical, will bind to the heme moiety of cytochrome P-450 (238).

The amino acid target(s) for BaP in microsomal proteins is unknown so far. BaP is metabolically activated to reactive epoxides, however, and these will conjugate readily with the SH group of GSH. This suggests that exposed SH groups might be involved in the binding. Lysine is another candidate, since metabolically activated BaP forms adducts with lysine residues in

histones (217). Most probably are these and other nucleophilic groups involved in adduct formation with microsomal proteins.

4.3. Metabolites involved in binding

Activated BaP metabolites responsible for DNA-binding have been investigated thoroughly (19,31). Several metabolites, including oxides, radicals, and secondary metabolites, have been shown to bind covalently. The metabolites bind to DNA to different extents, and they have different mutagenic and carcinogenic potencies. Because of these findings there has been intense search for the ultimate carcinogen(s) of BaP.

There have been few reports concerning the identity of BaP metabolites bound to proteins. Reactive epoxides and diolepoxides have been suggested to be binding species (7,30). This suggestion was supported by Raha et al. (251), who found that BaP-4,5-dihydrodiol was released by enzymatic and chemical hydrolysis of microsomal proteins with covalently bound BaP. This showed that K-region precursors were likely to be involved in the binding, and they suggested that BaP-4,5-epoxide was the ultimate binding species. Our studies, on the contrary, showed that BaP-4,5-epoxide only bound to microsomal proteins after metabolic activation (II). This finding suggests that secondary metabolites such as diolepoxides rather than BaP-4,5-epoxide are ultimate metabolites binding to microsomal proteins. Diolepoxides are also involved in the covalent binding of BaP to nuclear proteins (215).

In vitro experiments have shown that the presence of epoxide hydrolase and glutathione transferase will decrease the extent of binding of BaP to DNA (233,252). In reconstituted MFO systems there was less binding of BaP-7,8-dihydrodiol to proteins when epoxide hydrolase was present (235). We have shown that the cytosol has a similar effect on the binding of BaP to microsomal proteins (IV). Since epoxides are believed to bind to glutathione transferase (64) and epoxide hydrolase catalyzes the hydrolysis of epoxides (91), these findings suggest the involvement of epoxides in the binding to macromolecules. Many xenobiotics besides BaP have also been suggested to bind to proteins through epoxides, for instance PAHs (3,30), bromobenzene (165), chlorobenzene (172), DMBA (179), PCBs, (204), trans-stilbene (209), and aflatoxin B₁ (3,156). Involvement of an epoxide, primary or secondary, is also suggested by the decrease in binding found in the presence of GSH (I).

Other studies have shown that secondary products of phenolic metabolites rather than epoxides are the main binding species to microsomal proteins (160,196,206,253). One piece of evidence reported was that addition of UDPGA reduced the binding of xenobiotics, presumably due to conjugation of the phenolic metabolites. We also found a reduced protein binding when microsomes were incubated with BaP in the presence of UDPGA (I). Hesse and coworkers (206) proposed that the majority of bound dichlorobiphenyl originated from semiquinones or quinones. Tunek (254) suggested a possible metabolic pathway for benzene leading to protein binding, where benzene oxide, phenol and hydroquinones are intermediates with p-benzoquinone and/or p-benzosemiquinone as the main binding species. Quinones have also been suggested to be ultimate binding species for other compounds (159).

Radical formation is another possible route leading to protein binding. The reactive intermediates of CCl_4 are either trichloromethyl or trichloromethylperoxy radicals, which both are involved in macromolecular binding (228,238). Radical formation has also been suggested in the case of DMBA (255), since radical scavengers reduced covalent binding of this compound to proteins. It has been shown, however, that radical intermediates are not involved in the covalent binding of BaP-7,8-dihydrodiol to microsomal proteins (256).

The ultimate metabolite(s) of BaP that will bind covalently to microsomal proteins have not been identified yet, and the available information in literature is rather confusing. A few conclusions can be drawn, however, from our and the above studies. Several metabolites appear to be capable to bind as suggested by the results presented in paper II, and different metabolites have different target proteins. The binding species are probably secondary metabolites of BaP. Diol- and phenol-epoxides which bind to DNA are also likely to bind to proteins. An interesting alternative pathway was suggested by Jernström et al. (256), in which the diolepoxide undergoes another activation mediated by cytochrome P-450, before binding to microsomal proteins. Besides information about the ultimate binding species, it is of great interest to find out whether different protein-bound metabolites have different biological effects, as in the case of DNA binding.

4.4. Physiological significance

Covalent binding of xenobiotics to proteins may affect various biological functions in a cell. Several compounds, including acetaldehyde (149), CCl_4 (228,238), halothane (193), halobenzenes (165,172), paracetamol (151), parathion (237), PCBs (205,229), 1,1,2,2-tetrachloroethane (184), and thioacetamide (211), are cytotoxic causing liver necrosis. This has been correlated with covalent binding of their metabolites to cellular proteins. Other toxic effects reported are damage of bone marrow cells caused by benzene (160) and DMBA-dependent adrenal necrosis (257). A clear dose-dependent relationship between liver necrosis and covalent binding to proteins has been demonstrated with bromobenzene (165) and paracetamol (230).

Irreversible binding of xenobiotics to proteins seems to be highly specific in all cell fractions. Granted that there is a correlation between covalent protein binding and cytotoxicity, the toxic effect can therefore probably be attributed to the binding to a few crucial target proteins rather than to a more general binding. This was proposed already in the "protein-deletion" theory (135). Several enzyme activities had been found to decrease upon drug administration, which at that time was explained as covalent binding of the drugs, preferentially to sulfhydryl groups in enzymes (138,244). Later halothane (193) was found to inhibit several microsomal oxidizing reactions, an effect also seen with chloramphenicol (236) and parathion (237). The effects of the two latter compounds have been investigated thoroughly in reconstituted MFO systems. Thus, cytochrome P-450 undergoes a suicidal inactivation when these compounds become bound covalently to the cytochrome after metabolic activation catalyzed by the cytochrome itself. Several enzymes involved in RNA synthesis are sensitive to treatment with various carcinogens (148,214). In addition, BaP-7,8-dihydrodiol-9,10-epoxide has been found to bind to virus proteins and to inhibit the activity of reverse transcriptase (258).

In addition to their cytotoxic effects, numerous PAHs and other xenobiotics known to bind covalently to proteins cause transformation, mutation and cancer (31,84,259,260). An involvement of protein binding in carcinogenesis has not been established, but it has been suggested to be a likely step in tumor induction (225). Several facts support this suggestion, as outlined by Gronow (214). The genetic material is in intimate association with, and also protected by, proteins which control and regulate gene expression.

All known carcinogens that will bind to DNA also bind to proteins, and the protein binding is usually more extensive. Binding to biosynthetically active DNA regions has not been demonstrated. Finally, carcinogens bound to DNA can be removed by repair mechanisms. These facts suggest that DNA is not the exclusive macromolecular target in chemical carcinogenesis

Carcinogens have been demonstrated to influence RNA synthesis (148,214), and this could be involved in the carcinogenic process. Another observation that might be related to carcinogenicity is the covalent binding of BaP to histone H1 (217,261). BaP tends to bind to lysine residues in the histone, and this possibly leads to a restriction in the electrostatic interaction between these residues and the DNA backbone. This could explain the reduced affinity observed between BaP-modified histone H1 and DNA. Alternatively, such a modification may alter the conformation of the histone affecting histone-DNA interactions in a manner analogous to that proposed to occur after phosphorylation of histone H1 (261).

One physiological function of some intracellular proteins is to transport xenobiotics. Although such transport usually involves a noncovalent binding to the transporting protein, nothing argues against covalent binding in some instances. The cytosolic receptor proteins identified for BaP (262), DMBA (257), MC (263), and TCDD (264,265) bind these carcinogens noncovalently and transport them to the cell nucleus. There the carcinogens interact primarily with the Ah locus, the genetic site that controls the synthesis of cytochrome P-450 (265). A correlation between the level of inducibility of cytochrome P-450 and the amount of receptor has also been found (266). Interaction between these xenobiotics and nuclear proteins involved in gene regulation has also been proposed (263). Another cytosolic protein able to bind BaP noncovalently facilitates the transport of hydrophobic compounds such as BaP to cytochrome P-450 (267,268). This transport protein causes a more rapid metabolism of BaP, probably by preventing this hydrophobic molecule from getting embedded in the phospholipid bilayer of cellular membranes.

Evidence has been presented that activated azo carcinogens are transported within the cell embedded in a hydrophobic groove of a specific receptor protein (269). Thus the activated carcinogens are protected from reacting with cellular nucleophiles and may be transported to the cell nucleus where they can interact covalently with critical proteins. The binding

is described as being noncovalent, but is surprisingly firm withstanding SDS-gel electrophoresis and extraction with nonpolar solvents. This suggests that covalently bound xenobiotics also can be transported by proteins in the cell.

As to the covalent binding of PAHs to microsomal proteins, it is not known whether it has any physiological effects. Possible effects are that such binding is toxic, and that it can cause necrosis and enzyme inactivation. The proteins may be covalently modified in a suicidal manner, thereby acting as a "sink" for activated carcinogens to reduce the availability within the cell (216). This could prevent the nucleus, and in particular the DNA, from a covalent attack, leading to altered genetic information and cancer. The competition between different cell fractions able to bind xenobiotics favours this suggestion.

Possible effects on enzyme activities of covalently bound BaP has not been investigated yet. The observation that the binding of BaP to microsomal proteins still increases after prolonged incubation (III), together with our results that the binding still increases when most cytochrome P-450 molecules should have bound BaP (V), argues against an inactivation of cytochrome P-450 by covalently bound BaP. Other enzymes may be inactivated, however, after having bound BaP covalently. Studies of such possible effects should be of profound interest.

The endoplasmic reticulum interconnects different parts of the cell and there is a high lateral mobility of the membrane proteins which makes it possible, although yet speculative, for covalently modified microsomal proteins to act as transport proteins. Such a transport needs not necessarily be to the nucleus, as has been suggested (162), but can also be to the plasma membrane for further export of the ligand, or to lysosomes for its destruction as is supported by the finding that covalent binding to lysosomal components occurs later than to other subcellular fractions (224).

Another interesting observation in this connection (unpublished) that a few membrane-bound proteins dissociated from the membrane upon incubation of microsomes with BaP and NADPH. Some of these proteins were covalently modified by BaP. Kaderbhai et al. also found a deletion of a M_r 67 000 protein (270). This could be explained by a degradation of the protein following extensive covalent binding, a conformational change leading to

dissociation, or due to migration of the protein from the microsomal membrane.

4.5. Concluding remarks

In the present investigation we have tried to clarify some basic facts concerning the covalent binding of xenobiotics to microsomal proteins. Our results can be summarized as follows.

- * The covalent binding of xenobiotics to microsomal proteins is highly specific. Different binding patterns are seen with different xenobiotics. The binding patterns are highly influenced by pretreatment with inducers of drug-metabolizing enzymes. Metabolic activation is required for binding.
- * Different BaP metabolites have different protein target specificities. Secondary metabolites are the most likely molecular species to bind.
- * Cytosolic proteins reduce the binding of BaP to microsomal proteins. BaP binds covalently to two cytosolic polypeptides, which appear to be subunits of glutathione transferase.
- * Microsomal targets for BaP are isozymes of cytochrome P-450 and epoxide hydrolase, but not NADPH-cytochrome P-450 reductase.
- * A rapid and sensitive method has been developed for the quantitative determination of covalent binding of xenobiotics to proteins.

There are as yet several unidentified target proteins in the microsomes. Two targets of particular interest is the highly labelled component of M_r 60 000 in PB microsomes that binds BaP metabolites activated in the 4,5-position, and the 70 000 M_r protein which, in particular, binds benzene, chlorobenzene and phenol. Besides the identification of target proteins, studies as to the effect of covalent binding on their function is of profound interest. Other interesting questions concern the identity of target nucleophilic sites in proteins and the identification of ultimate binding species of BaP.

I believe that it is of central importance to determine the effect on the cell function of the covalent binding of xenobiotics to cellular proteins. The binding is most likely involved in cytotoxicity, causing necrosis and enzyme inactivation. It has not been demonstrated that covalent binding is involved in the carcinogenic process, although the cooperation between proteins and DNA indicates covalent binding to be important in this process. Further studies on the covalent binding of xenobiotics to proteins may be important for the development of biological exposure tests for carcinogens (250). Biological monitoring can perhaps be based on the determination of amino acid adducts, since they are more abundant in vivo than DNA adducts.

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Coelia Colvelin

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MICROSOMAL TARGET PROTEINS OF METABOLICALLY ACTIVATED AROMATIC HYDROCARBONS

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SUMMARY

The specificity of binding to microsomal proteins of metabolically activated hydrocarbons has been studied. Radioactively labelled benzene, phenol, chlorobenzene, BP and MC were incubated with liver microsomes from control, phenobarbital- and MC-treated rats in the presence of an NADPH-generating system. The patterns of metabolite binding to microsomal proteins were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography. Benzene, phenol and chlorobenzene metabolites showed one type of binding pattern dominated by a band at 72 000 M_r . This band was strong both in control and induced microsomes. Additional radioactive bands were seen in the 50 000–60 000 M_r region particularly in MC-induced microsomes. BP and MC metabolites showed a different type of binding pattern with incorporation of radioactivity into several fractions in the 50 000–60 000 M_r region of MC-induced microsomes. Two other strongly labelled fractions occurred at 68 000 and 72 000 M_r . The incorporation was low into control and phenobarbital-induced microsomes. Two labelled bands (M_r 56 000 and 72 000) were common for all hydrocarbons in MC-induced microsomes. The 56 000 M_r band had the same mobility in the gel as an MC-induced protein likely to be cytochrome P-448. The NADPH-generating system was essential for metabolite binding and GSH and UDPGA greatly reduced binding. We suggest that differences in metabolite binding patterns reflect differences in the routes of metabolite formation and that activated hydrocarbons are likely to bind to proteins close to their site of formation.

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Abbreviations: BP, benzo[a]pyrene; GSH, reduced glutathione; MC, 3-methylcholanthrene; SDS, sodium dodecylsulfate; UDPGA, UDP-glucuronic acid.

INTRODUCTION

Many toxic and/or carcinogenic compounds exert their adverse effects only after metabolic activation [1]. The reactive metabolites thus formed can bind covalently to cellular macromolecules and disturb normal cellular functions, giving rise to cytotoxic effects, mutation or cancer [2,3]. Most such compounds are metabolised by the mixed-function oxidase system located principally in the endoplasmic reticulum.

There has been a particularly intense interest in the covalent binding of activated hydrocarbons to DNA, since it seems reasonable to assume that carcinogenic and mutagenic effects originate from such interactions. However, covalent binding to microsomal macromolecules by metabolites of a large number of compounds has also been demonstrated after incubation with microsomes [4]. Metabolites of some compounds, e.g. 4-chlorobiphenyl, bind preferentially to ribosomal RNA [5], and others, such as benzene, bind almost exclusively to proteins [6]. Massive covalent binding to macromolecules is thought to contribute to cytotoxicity, as suggested for e.g. bromobenzene [3].

While there has been a great interest in the binding of metabolites to a variety of macromolecules, there have been no reports on the specificity of binding to proteins at the site of activation, i.e. to microsomal proteins. In this study we have incubated radioactively labelled hydrocarbons with rat liver microsomes and examined the specificity of metabolite binding to microsomal proteins by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

MATERIALS AND METHODS

All chemicals used were of analytical grade. [^{14}C]Benzene, [^{14}C]phenol, [^{14}C]BP and [^3H]MC were from the Radiochemical Centre, Amersham.

Male Sprague-Dawley rats, weighing approx. 150 g, were obtained from Anticimex, Stockholm. They were maintained on a standard chow diet and free access to water. Phenobarbital, 80 mg/kg, was injected i.p. on three consecutive days and the animals were sacrificed on the fourth day. MC, 80 mg/kg in corn oil, was injected i.p. 24 h before sacrifice. Liver microsomes were prepared as described earlier [6]. Protein was determined by the Lowry procedure [7] using bovine serum albumin as standard.

The incubation mixture used for studies of hydrocarbon binding to microsomal macromolecules contained 0.6 ml of buffer (100 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 and 10 μM MnCl_2), an NADPH-generating system (2 mg glucose 6-phosphate, 1.5 mg NADP and 2.7 μg (37 U) glucose-6-phosphate dehydrogenase), radiolabelled hydrocarbons and 1.2–1.8 mg microsomal protein in a total volume of 1.2 ml. The hydrocarbons were added dissolved in 10 μl acetone in amounts and specific activities as indicated in each case. The incubations were carried out at 37°C for 60 min, unless otherwise is stated. When used, GSH or UDPGA were administered

dissolved in 100 μ l of water to give final concentrations of 2 mM and 15 mM, respectively.

The quantitative determination of irreversible binding to microsomal macromolecules was performed as described earlier [6], except that the final protein pellet was dissolved in 0.5 ml Soluene-350 (Packard). A 0.2-ml aliquot of the protein solution was counted in 10 ml Dimilume-30 (Packard).

SDS-polyacrylamide gel electrophoresis was performed as described in detail elsewhere [8,9]. After incubation, 100 μ l aliquots of the incubation mixture (100–150 μ g of microsomal protein) were mixed with 100 μ l of a denaturing medium containing SDS [9] and boiled for 2 min. After cooling 100 μ l was applied to 11% SDS-polyacrylamide slab gels. The electrophoresis was run until the visible salt front reached the bottom of the gel. Gels were stained for 15 h in 0.1% Coomassie Brilliant Blue dissolved in 50% methanol and 7% acetic acid and destained electrophoretically in 25% methanol and 7% acetic acid. The gels were treated with PPO and dried [10] before fluorography. Kodak X-Omat films were exposed to the gels at -70°C for 1–6 weeks. Molecular weight standards used were bovine serum albumin ($M_r = 69\,000$), ovalbumin ($M_r = 45\,000$), soybean trypsin inhibitor ($M_r = 21\,500$) and sperm whale myoglobin ($M_r = 17\,200$).

RESULTS

Time-course and extent of metabolite binding to microsomes. When aromatic hydrocarbons are incubated with rat liver microsomes in the presence of an NADPH-generating system reactive metabolites are formed that will bind irreversibly to microsomal components. The time-dependent binding of phenol, BP and MC metabolites to microsomes from MC-induced rat livers was studied at a hydrocarbon concentration of 50 μM (Fig. 1). The extent of irreversible metabolite binding was the highest for phenol, and 2.8 nmol had bound per milligram membrane protein after 60 min of incubation corresponding to approx. 5% of the total amount of hydrocarbon added. BP and MC metabolites bound to a much smaller extent, 0.8 and 0.6 nmol/mg membrane protein corresponding to 1.5% and 1.2% of the added hydrocarbon. The binding of hydrocarbon metabolites to control and phenobarbital induced microsomes followed similar time-courses as in MC-induced microsomes. The extent of binding of MC and BP metabolites was lower, however; than in MC-induced microsomes (cf. Fig. 2). Phenol metabolite binding was less affected by inducers and the control microsomes even showed a higher binding than MC-induced microsomes (4 vs. 2.8 nmol/mg protein after 60 min of incubation). The extent of metabolite binding also appeared to be dependent on the substrate concentration used. More thorough studies on the quantitative aspects of metabolite binding are underway, where we examine the dependency on substrate concentration and the extent of metabolite binding to individual proteins.

The binding of all hydrocarbons examined was entirely dependent on the presence of an NADPH-generating system. It is clear therefore, that binding

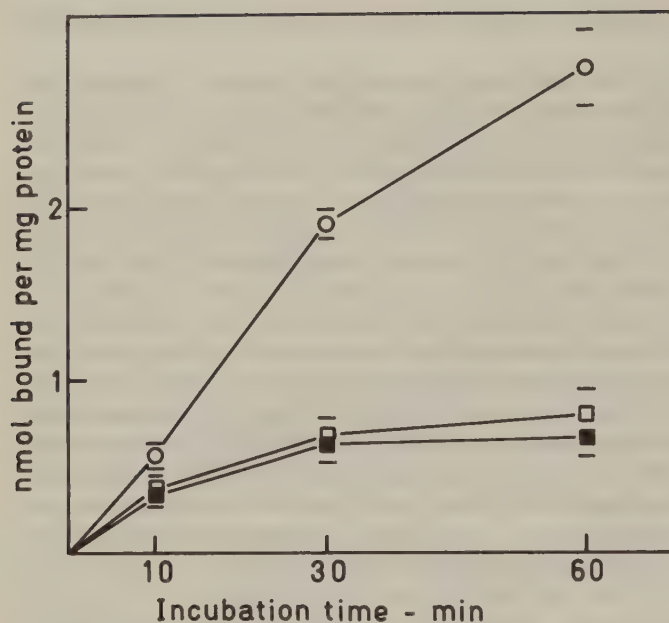
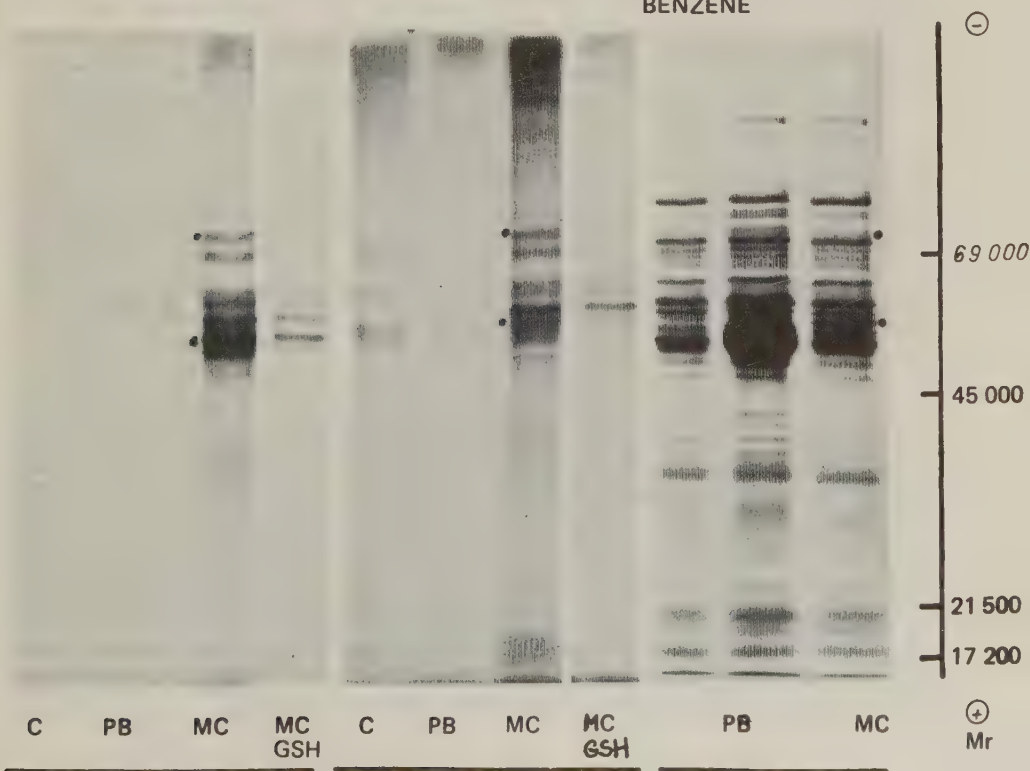
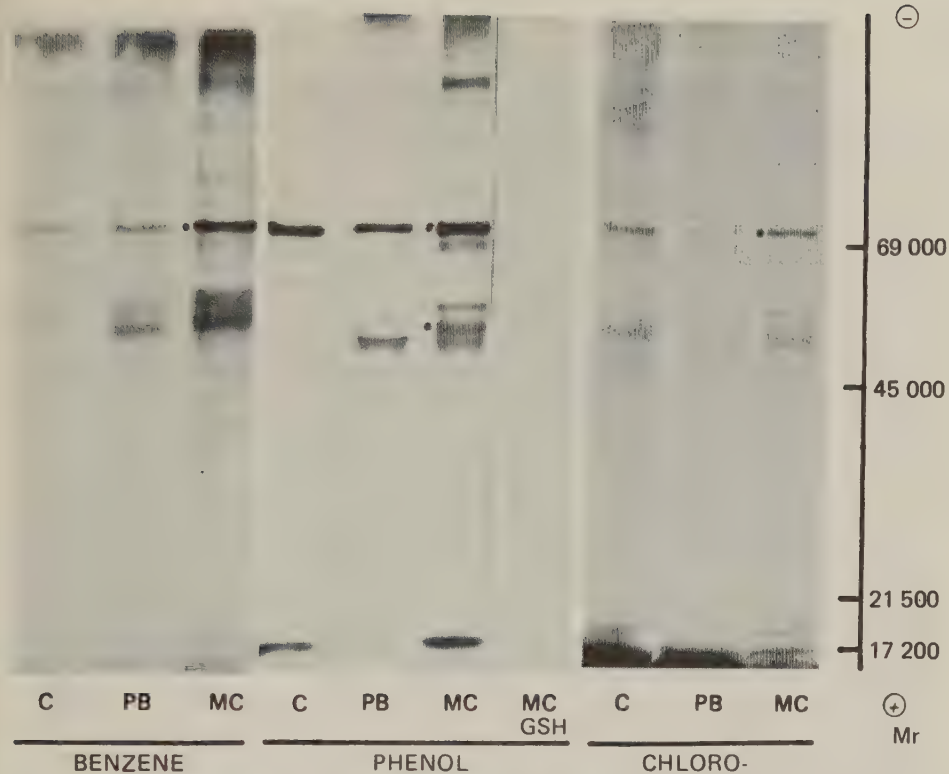


Fig. 1. Quantitative determination of the irreversible binding of metabolites of phenol (○), BP (□) and MC (■) to liver microsomes from MC-pretreated rats. The results are based on experiments with three animals and standard deviations are indicated. The amount of substrate was 60 nmol and the specific activities were for phenol 5.2 mCi/mmol, BP 13 mCi/mmol and MC 81 mCi/mmol. Microsomal protein (1.2 mg) was used in each incubation. Zero time incubations (<3% of the binding observed after 60 min) have been subtracted.

was preceded by a metabolic activation of the hydrocarbons to reactive metabolites.

Metabolite binding patterns to microsomal proteins. To examine whether the irreversible binding of metabolites to microsomal proteins occurred selectively or randomly, liver microsomes from control, phenobarbital and MC pretreated rats were incubated with radiolabelled aromatic hydrocarbons in the presence of an NADPH-generating system. The binding patterns of the metabolites were obtained by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 2).

Fig. 2. Hydrocarbon metabolite binding patterns and protein patterns of control and induced rat liver microsomes. Microsomes were incubated as described under Materials and Methods with one of the following hydrocarbons dissolved in 10 μ l acetone: [14 C]phenol, 13 nmol (35 mCi/mmol); [14 C]chlorobenzene, 417 nmol (5 mCi/mmol); [14 C]BP, 23 nmol (60.7 mCi/mmol); [3 H]MC, 0.92 nmol (9 Ci/mmol). After incubation samples were prepared for electrophoresis as described in Materials and Methods. C, PB and MC indicate control, phenobarbital- and methylcholanthrene-induced microsomes, respectively. The incubations contained 1.4 (C), 1.8 (PB) or 1.5 (MC) mg of microsomal protein. Metabolite binding patterns to MC-induced microsomes were also examined in presence of 2 mM GSH. The 56 000 and 72 000 M_r fractions specifically referred to in the text are indicated by dots.



The binding patterns of benzene, phenol and chlorobenzene metabolites were quite similar. Their most striking feature was the dominant radioactive band with an $M_r = 72\,000$ that was particularly conspicuous in the phenol incubations. Additional labelled bands occurred in the $50\,000$ – $60\,000\ M_r$ region. Phenol and chlorobenzene metabolites also bound extensively to a component in the high molecular weight region and to a component with an $M_r = 19\,000$. The relative density of the radioactive bands was affected by the microsome inducing system, with the most intense bands in the $50\,000$ – $60\,000\ M_r$ region occurring in the MC-induced microsomes.

The binding patterns for BP and MC metabolites were distinctly different from those of benzene, phenol and chlorobenzene. The enhancement in binding to MC-induced microsomes was particularly striking at the hydrocarbon concentrations used in this experiment. Most of the binding to these microsomes was concentrated in at least three bands in the $50\,000$ – $60\,000\ M_r$ region. One band at $68\,000\ M_r$ was rather prominent after incubation with BP and MC when compared to the other hydrocarbons. Two bands with $M_r\ 56\,000$ and $72\,000$ (indicated by dots in Fig. 2) were seen for all the hydrocarbons. The former occurred after MC-induction only and had the same mobility as a protein component induced by MC treatment which most likely is cytochrome *P-448* [11].

The presence of an NADPH-generating system was an absolute requirement for metabolite binding. When reduced glutathione was included in the incubation medium the binding of benzene, phenol and chlorobenzene metabolites was almost completely prevented (the inhibition was approx. 95%) while the binding of BP and MC metabolites was inhibited by around 65%.

The microsomal protein patterns are also shown in Fig. 2. The $56\,000\ M_r$ band and the position of the $72\,000\ M_r$ radioactive band have been marked by dots.

Effect of substrate concentration on metabolite binding patterns. The results presented in Fig. 2 were obtained with various hydrocarbon concentrations in the incubation medium. To examine the influence of substrate concentration on binding patterns microsomes from MC-induced rats were incubated with four concentrations of each of the substrates phenol, BP and MC. The metabolite binding patterns from this experiment are shown in Fig. 3. In the case of phenol most binding occurred to the $72\,000\ M_r$ component at the lowest substrate concentration tested. At higher phenol concentrations, however, this band was less dominant, and the major binding components in the $50\,000$ – $60\,000\ M_r$ region then showed a comparable extent of metabolite binding. The binding pattern of BP metabolites was not influenced significantly by the substrate concentration (there was a faint pattern at the highest concentration due to the low specific radioactivity that had to be used). The binding pattern of MC metabolites was dominated by the incorporation into the $56\,000\ M_r$ component at the lowest substrate concentration tested. At higher substrate concentrations, however, the incorporation occurred predominantly to two other components in the $50\,000$ – $60\,000\ M_r$ region.

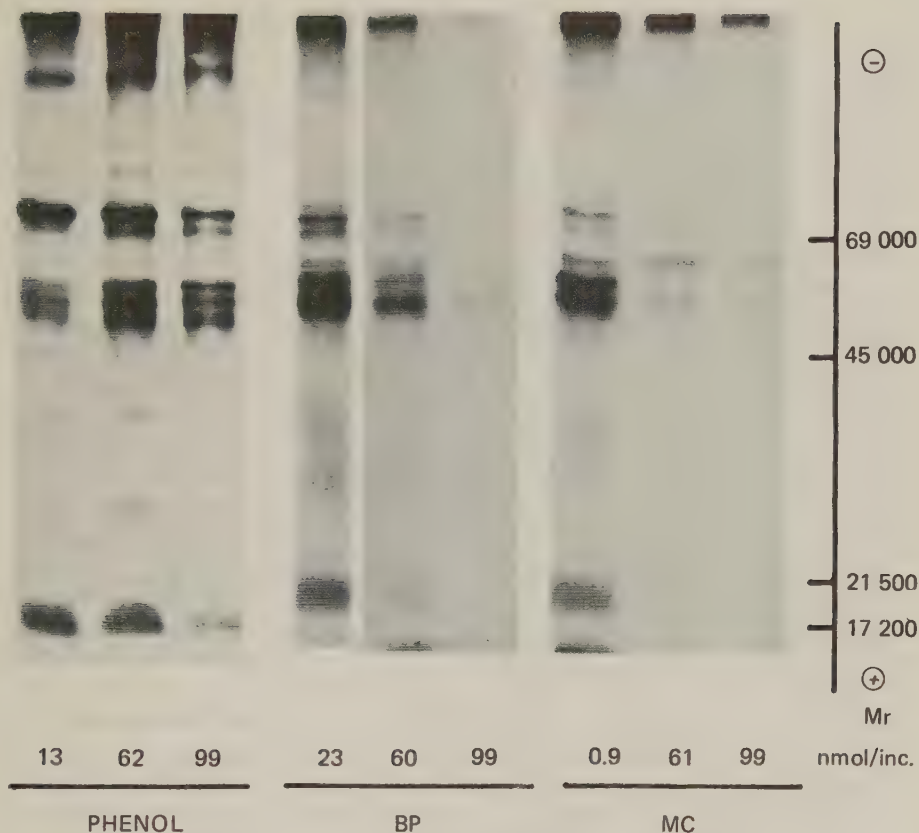


Fig. 3. Effect of substrate concentration on the binding patterns of phenol, BP and MC metabolites. Microsomes (1.4 mg) from MC-treated rats were incubated with the following amounts of substrate dissolved in 10 μ l acetone: phenol, 13 nmol (35 Ci/mmol), 62 nmol (7.6 mCi/mmol) and 99 nmol (4.7 mCi/mmol); BP, 23 nmol (61 mCi/mmol), 60 nmol (23 mCi/mmol) and 99 nmol (14 mCi/mmol); MC, 0.92 nmol (9 Ci/mmol), 61 nmol (136 mCi/mmol) and 99 nmol (84 mCi/mmol). After incubation electrophoresis and autoradiography were performed as described in Materials and Methods.

Effect of incubation time and UDPGA on metabolite binding patterns. The influence of incubation time on the binding patterns of phenol, BP and MC metabolites to liver microsomes from MC-induced rats was also examined (Fig. 4). The binding patterns for phenol and BP metabolites did not change appreciably with incubation time. In the case of MC metabolites, however, the band at 56 000 M_r was rather faint after 10-min incubation, but it became very prominent after 60 min. The presence of UDPGA, which will conjugate with metabolites containing hydroxy groups decreased the intensity of all bands in the BP and MC metabolite patterns (Fig. 4). This implies that at least a part of the binding to each band in the absence of

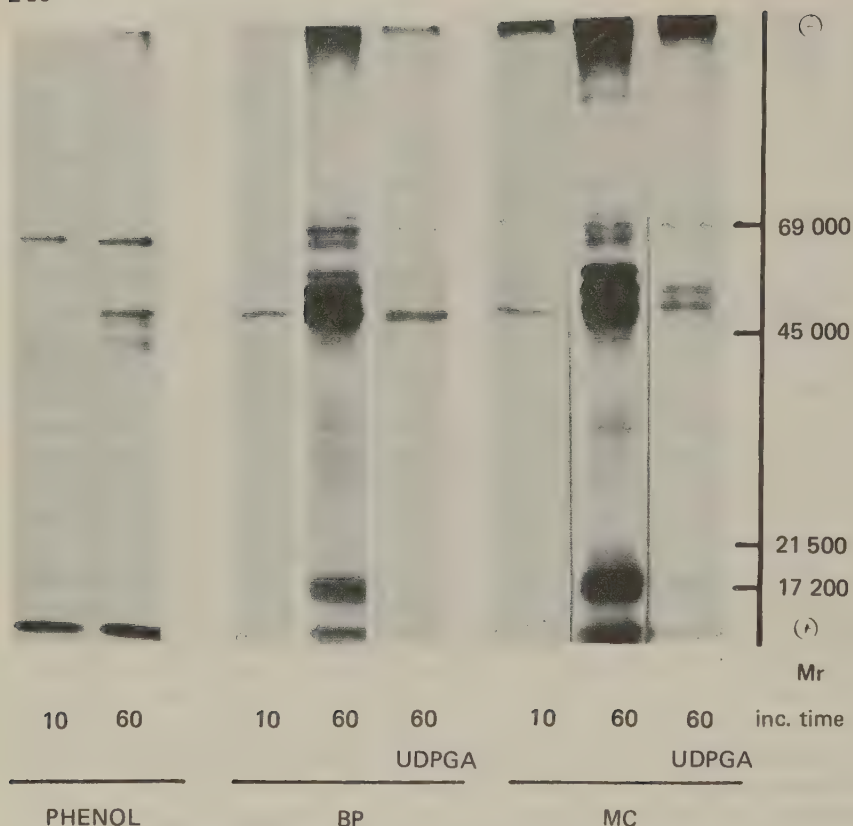


Fig. 4. Effect of incubation time and UDPGA on the binding patterns of phenol, BP and MC metabolites to MC-induced microsomes. The hydrocarbons were used at the lowest concentrations and corresponding specific radioactivities as given in the legend to Fig. 3. Incubation times were 10 min and 60 min. Microsomal protein (1.4 mg) was used in each incubation. UDPGA was used at a concentration of 15 mM. After incubation electrophoresis and autoradiography were performed as described in Materials and Methods.

UDPGA was due to secondary metabolites. The binding of phenol metabolites in the presence of UDPGA was not tested since phenol itself would conjugate with this compound.

DISCUSSION

The potential importance of hydrocarbon metabolite binding to protein for the generation of cell toxicity has been recognized for several years [4]. As far as we know, however, a direct link between metabolite binding to specific proteins in the cell and cell toxicity has not been established.

Our results show that in isolated microsomes there is a preferential

binding of hydrocarbon metabolites to a small set of proteins. It is possible, therefore, that if there is a link between metabolite binding and cell toxicity, this might be due to specific rather than random interactions between metabolites and proteins.

In order to bind to proteins the toxic and/or carcinogenic hydrocarbons tested had to be metabolically activated as indicated by the absolute requirement of an NADPH-generating system for binding. The activation produces electrophilic intermediates which then react with nucleophilic sites in macromolecules [12–14]. Nucleophilic binding sites in proteins include the sulfhydryl group of cysteine. It has been shown earlier that metabolically activated MC and 7,12-dimethylbenzanthracene most likely bind to proteins via such sulfhydryl groups [15]. A similar type of binding, at least for the major part of the ultimate binding metabolites of the hydrocarbons examined here, is suggested by the inhibition of binding by reduced glutathione.

The most extensive binding was observed for phenol metabolites of which more than 2.5 nmol bound irreversibly per milligram of microsomal protein. Since the binding occurred preferentially to one protein fraction ($M_r = 72\,000$), several phenol metabolites appear to have bound to each protein molecule. An exact determination of the binding stoichiometry has to await the isolation of the binding protein. The experiments with increasing concentrations of phenol in the incubation medium or longer incubation times showed that other protein components also would bind phenol metabolites under such conditions. It is likely that the potential binding sites of the 72 000 M_r protein then became saturated and that excess metabolites reacted with proteins which were normally less prone to bind metabolites. Metabolites of the other hydrocarbons bound to a much smaller extent and no saturation was observed in these cases.

Since the hydrocarbon metabolites appeared to bind specifically rather than randomly to microsomal proteins and since different protein binding patterns were observed for different hydrocarbons the question arises which factors will determine the specificity of metabolite binding. One possibility is that the kind of ultimate binding metabolite formed (for instance a semi-quinone or an epoxide) determines the specific target protein(s). Another possibility is that the metabolite will bind to any macromolecule with an exposed reactive group. The latter suggestion implies that any newly formed reactive metabolite most likely will bind to a reactive protein close to the site of metabolite formation. Thus, the enzyme that catalyses the formation of the metabolite would itself receive most of the binding provided it contains an exposed reactive group. A matter in point is the radioactive band of 56 000 M_r which is obtained after incubation of all five hydrocarbons with MC-induced microsomes. This band has the same mobility in the gel as a protein fraction induced by MC which is likely to be cytochrome *P*-448. However, it is not possible from this observation alone to resolve which factor determines the specificity of metabolite binding, particularly since the complete metabolic conversion pathways for the various hydrocarbons remain unclear.

Either of the suggested mechanisms for binding specificity permits the interpretation that the similarities in metabolite binding patterns for benzene, phenol and chlorobenzene depend on similar activation pathways for these compounds. The ultimate binding metabolites for phenol and benzene are probably mainly quinones and semiquinones [6; A. Tunek, K.L. Platt and F. Oesch, unpublished). Chloro- and bromobenzene have both been thought to be activated by the mixed-function oxidase system to epoxides which would bind to macromolecules causing hepatotoxic effects [16–18]. Provided that there is a correlation between the type of ultimate binding metabolite and binding pattern, our results agree more with the recent suggestion [19], that at least a part of the protein binding metabolites of bromobenzene originate through a further metabolism of the hydroxylated compound to a semiquinone or a quinone.

The metabolic activation of BP and the subsequent binding to DNA has been studied in great detail. Dihydrodiol epoxides and further oxidized BP-phenols (mainly BP-9-hydroxy-4,5-oxide) seem to be the main binding metabolites produced by MC-induced microsomes [20,21]. Less is known about the metabolic activation of MC. Recent data indicate, however, that several dihydrodiol epoxides can be formed [22,23]. The binding patterns for BP and MC were rather similar, but they were distinctly different from those of benzene, phenol and chlorobenzene. Thus, the available data on the activation pathways of the compounds examined and the binding patterns presented here suggest that differences in the mechanism of metabolic activation also are reflected in differences in binding patterns.

The influence of substrate concentration on the binding pattern of MC-metabolites is interesting. At the lowest concentration used a band at 56 000 M_r is clearly dominating, but at higher MC-concentrations this band is very faint. Figure 4 shows the influence of incubation time on the binding patterns of phenol, BP and MC-metabolites at a substrate concentration identical to the lowest one in Fig. 3. Here the metabolite pattern is dominated by the 56 000 M_r band only after the longest incubation time. These observations suggest that the binding of MC metabolites to the 56 000 M_r protein may require more metabolic steps than the binding to other proteins.

In summary, the findings presented in this paper suggest that there are differences in binding patterns of activated hydrocarbons to microsomal proteins and that the pattern is dependent on the type of metabolite which is the ultimate binding agent. Of interest may now be to isolate and identify metabolite target proteins in order to examine the influence of metabolite binding on their biological function and the possible role of binding for the development of cytotoxicity.

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Irreversible Binding of Isolated Benzo[a]pyrene Metabolites to Specific Rat Liver Microsomal Proteins

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SUMMARY

SCHELIN, C., A. TUNEK, B. JERNSTRÖM AND B. JERGIL. Irreversible binding of isolated benzo[a]pyrene metabolites to specific rat liver microsomal proteins. *Mol. Pharmacol.* 18: 529-535 (1980).

[³H]Benzo[a]pyrene and seven isolated [³H]benzo[a]pyrene metabolites were incubated with liver microsomes from control, phenobarbital-, and 3-methylcholanthrene-treated rats, and irreversible binding to microsomal proteins was studied. For all compounds tested, including benzo[a]pyrene-4,5-oxide, the binding was greatly enhanced in the presence of a NADPH-generating system. All metabolites except 3-hydroxybenzo[a]pyrene bound more extensively to microsomal proteins from 3-methylcholanthrene-treated rats than to proteins from phenobarbital-treated or control rats. Benzo[a]pyrene-7,8-dihydrodiol bound much more efficiently than the other metabolites. The protein binding patterns of the metabolites were examined by SDS-polyacrylamide gel electrophoresis and fluorography. The most extensive binding occurred to a few proteins in the MW region of 45,000-70,000, and there were differences in the patterns between different metabolites. The composite pattern of the metabolites corresponded to the binding pattern obtained with benzo[a]pyrene. A major target protein in microsomes from 3-methylcholanthrene-treated rats had the same mobility as purified cytochrome P-448. Most binding to microsomes from phenobarbital-treated animals occurred to a 60,000 MW component. Binding also occurred to a protein which comigrated with cytochrome P-450. The specificity and extent of metabolite binding to proteins may be of importance for the development of cytotoxicity. Factors affecting the binding patterns are discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PCH) are primarily metabolized by the cytochrome P-450-linked monooxygenase system located mainly in the endoplasmic reticulum. The result of the metabolism is more polar products such as phenols, dihydrodiols, or further oxygenated compounds which become water soluble and readily excretable after conjugation. During this metabolism reactive intermediates are formed which may bind covalently to cellular macromolecules (1, 2).

BP is the most thoroughly studied PCH, and it is

metabolized to several products in rat liver (3). The metabolite pattern obtained depends on the state of activation of the inducible cytochrome P-450-linked monooxygenase system. Phenobarbital, for instance, induces certain species of cytochrome P-450 and, thereby, a preferential metabolism in the 4,5 region of the BP molecule (4). MC, on the other hand, induces a different form of the cytochrome (cytochrome P-448) which increases the metabolic rate of BP and shifts the metabolism to the 7,8 and 9,10 region of the molecule (4, 5). BP-7,8-dihydrodiol is considered to be a proximate carcinogen of BP (6) and is converted to the ultimate carcinogen by further oxygenation in the 9,10 region to isomeric BP-7,8-dihydrodiol-9,10-oxides (7, 8). These may bind covalently to DNA (9).

There are several reports on the binding of PCH and PCH metabolites to DNA. Much less is known of their binding to cellular proteins, and in particular to microsomal proteins, i.e., at the site of metabolic activation. However, several toxic and carcinogenic compounds including BP have been shown to bind covalently to micro-

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¹ Abbreviations used: BP, benzo[a]pyrene; 3-OH-BP, 3-hydroxybenzo[a]pyrene; 9-OH-BP, 9-hydroxybenzo[a]pyrene; BP-4,5-dihydrodiol, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene; BP-7,8-dihydrodiol, 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene; BP-9,10-dihydrodiol, 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene; BP-4,5-oxide, benzo[a]pyrene-4,5-oxide; BP-3,6-quinone, benzo[a]pyrene-3,6-quinone; MC, 3-methylcholanthrene; PCH, polycyclic aromatic hydrocarbons.

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somal proteins after metabolic activation (10). We have found that BP, MC, and the toxic aromatic compounds phenol, benzene, and chlorobenzene bind irreversibly and specifically to a few microsomal proteins after metabolic activation (11). Although the major DNA-binding species of BP are further activated primary metabolites, there is no information available on the binding of such metabolites to microsomal or other proteins. We have examined the incorporation of BP and individual BP metabolites to microsomal proteins.

MATERIALS AND METHODS

Chemicals. [^3H]BP (19 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. [^3H]BP-4,5-oxide was a generous gift from Dr. Franz Oesch, Mainz. It was also obtained from the National Cancer Institute Carcinogenesis Research Program, Bethesda, Md. Isotopically labeled BP metabolites (3-OH-BP, 9-OH-BP, BP-4,5-dihydrodiol, BP-7,8-dihydrodiol, BP-9,10-dihydrodiol, and BP-3,6-quinone) were obtained by incubating [^3H]BP with liver microsomes from MC-treated rats. The metabolites were isolated as described by Jernström *et al.* (12). They were pure according to high-pressure liquid chromatography and absorption spectra when compared to authentic BP metabolites. All other chemicals used were of analytical grade.

Animals and treatments. Male Sprague-Dawley rats (Anticimex, Stockholm), weighing 150–200 g, were allowed food and water *ad libitum*. Phenobarbital (80 mg/kg) was injected intraperitoneally on 3 consecutive days and the rats were killed on the fourth day. MC (80 mg/kg in corn oil) was injected once intraperitoneally 24–35 h before sacrifice. Control animals were untreated, but we have ascertained in other experiments that the injection of saline or corn oil does not affect the results.

Preparation of microsomes. The rats were stunned by a blow on the head and bled to death by severing the carotid artery. Livers were excised immediately, chilled, and homogenized in 2.5 ml of buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 25 mM KCl, 0.35 M sucrose) per g of tissue using a Potter-Elvehjem homogenizer with a Teflon pestle. Microsomes were obtained by sedimenting the postmitochondrial (10,000g for 10 min) supernatant at 105,000g for 90 min. The pellet was washed once by suspension in the buffer and sedimentation as above. The final pellet was suspended in buffer without sucrose at a protein concentration of 8–12 mg/ml, and aliquots were stored under N_2 at -70°C .

Analytical procedures. Protein concentrations were determined by the procedure of Lowry *et al.* (13) using bovine serum albumin as the standard. Cytochrome P-450 was determined according to Omura and Sato (14). The average cytochrome P-450 concentrations were 0.7 nmol/mg protein in control microsomes, 2.4 nmol/mg protein after induction with phenobarbital, and 1.2 nmol/mg protein after induction with MC.

Quantitative determination of irreversible metabolite binding to proteins. The binding of BP or isolated BP metabolites to microsomal proteins was performed in the presence of a NADPH-generating system as previously described in detail (11). The quantitative binding was

determined after extractions as described in (15). BP or BP metabolites were added to the incubation mixture dissolved in 10 μl of methanol, except for BP-3,6-quinone and BP-4,5-oxide, which were added in 10 μl of dimethyl sulfoxide. Amounts and specific activities of the substrates and the incubation times used in different experiments are indicated in each case.

Analysis of protein binding patterns by electrophoresis. Samples containing 100 μg of membrane protein obtained from the incubations were treated for SDS-polyacrylamide gel electrophoresis and electrophoresed in slab gels as described earlier (11, 16, 17). After staining and destaining, gels were treated with 2,5-diphenyloxazole (18) before fluorography. Kodak X-Omat films were exposed to the gels at -70°C for 1–6 weeks.

RESULTS

Metabolic activation is required for irreversible metabolite binding. Appreciable irreversible binding of BP or its metabolites to microsomal proteins occurred only after metabolic activation. Incorporation of metabolites to proteins in liver microsomes from phenobarbital- or MC-treated rats in the absence of NADPH was below 10% of the binding obtained in the presence of a NADPH-generating system. In microsomes from control rats, where the incorporation was comparatively low for most of the metabolites tested, the corresponding figure was approximately 20%. BP-4,5-oxide, which in itself is supposed to be a reactive intermediate, did not bind more than the other metabolites in the absence of the NADPH-generating system.

Time course of metabolite binding. The irreversible binding of BP and seven BP metabolites to liver microsomes from control, phenobarbital-, and MC-treated rats after metabolic activation was studied as a function of time at a substrate concentration of 0.8 μM (Fig. 1). The extent of binding was highest to microsomes from MC-treated rats for all substrates tested except for 3-OH-BP, which initially bound more efficiently to microsomes from phenobarbital-treated rats. BP-7,8-dihydrodiol bound most extensively of all substrates examined to microsomes from MC-treated animals. This reaction appeared to be comparatively rapid, since the binding was complete within the first 10 min of incubation. In contrast, the binding of the other substrates progressed with a significant rate for at least 30 min. The time-dependent binding was also examined at a substrate concentration of 8 μM (not shown). The incorporation curves were very similar to those obtained at 0.8 μM , except that the incorporation was more extensive (cf. Fig. 2).

Effect of metabolite concentration on binding. The irreversible binding of BP metabolites to microsomal proteins after further activation was examined at various metabolite concentrations (Fig. 2). For most metabolites the extent of binding to microsomes from both treated and untreated animals increased roughly linearly with increasing substrate concentrations.

At all substrate concentrations examined the binding to microsomes from MC-treated rats was quantitatively higher than the binding to microsomes from control or phenobarbital-treated rats. An exception was 3-OH-BP,

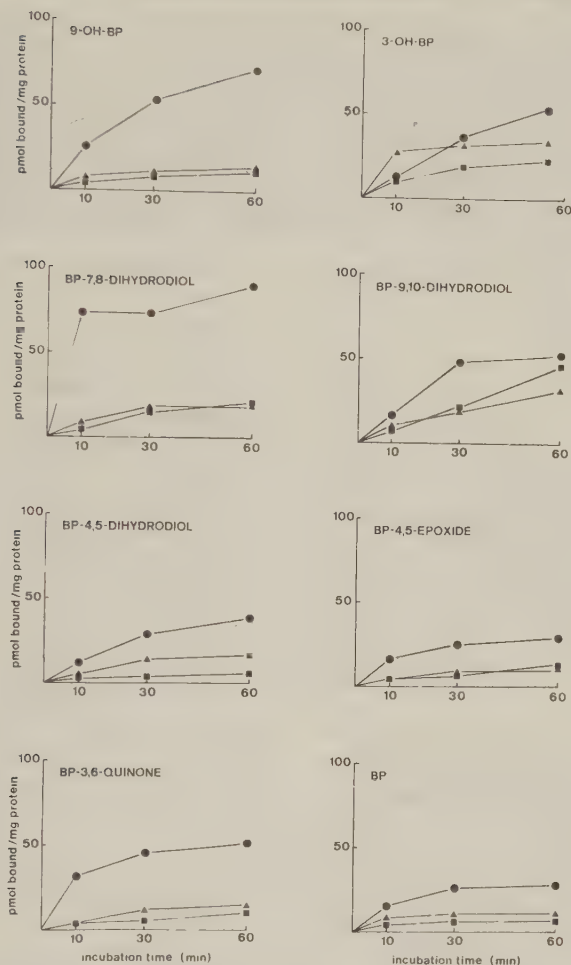


FIG. 1. Quantitative determination of the irreversible binding of BP and BP metabolites to liver microsomes from control (■), phenobarbital (▲), and MC (●)-pretreated rats.

Incubations were performed as described in the experimental section using a substrate concentration of $0.8 \mu\text{M}$ and the following specific activities: BP, 19 Ci/mmol; 3-OH-BP, 0.84 Ci/mmol; 9-OH-BP, 0.66 Ci/mmol; BP-7,8-dihydrodiol, 0.53 Ci/mmol; BP-9,10-dihydrodiol, 0.47 Ci/mmol; BP-4,5-dihydrodiol, 0.54 Ci/mmol; BP-4,5-oxide, 0.44 Ci/mmol; and BP-3,6-quinone, 1.32 Ci/mmol.

which bound preferentially to microsomes from phenobarbital-treated animals. Of the compounds tested, BP-7,8-dihydrodiol was activated to products that bound more extensively to microsomes from MC-treated rats (1.1 nmol incorporated/mg membrane protein) than the products from the other compounds (less than 0.4 nmol). The incorporation into microsomes from phenobarbital-

treated or control animals was 0.1 nmol/mg protein or less. In no case was a saturation in metabolite binding observed. Concentrations exceeding $8 \mu\text{M}$ were not tested due to a limited supply of metabolites. However, we have tested the incorporation of BP up to a concentration of $50 \mu\text{M}$ without obtaining a saturation in the binding.

Binding patterns to microsomal proteins. The protein

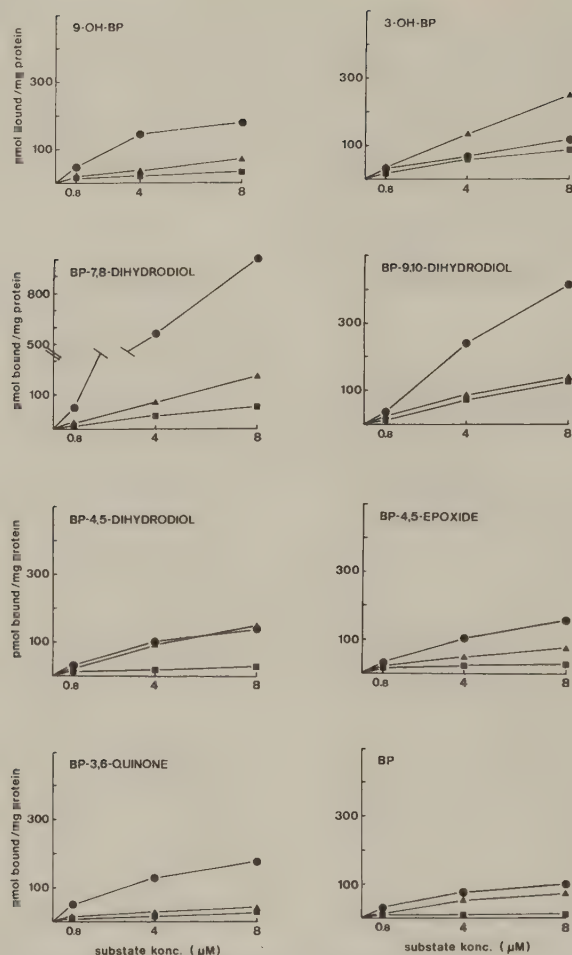


FIG. 2. Effect of substrate concentration on the irreversible binding of BP and BP metabolites to liver microsomes from control (■), phenobarbital (▲), and MC (●)-pretreated rats

Incubations were performed as described in the experimental section using three substrate concentrations. The incubation time was 30 min. The specific activities at 0.8 μM were the same as in Fig. 1. At 4 μM they were reduced to 1/10, except for BP-4,5-oxide and BP-3,6-quinone, which had the same specific activities as at 0.8 μM . The specific activities at 8 μM were (given in the same order as in Fig. 1.) 0.95, 0.042, 0.033, 0.027, 0.024, 0.027, 0.44, and 1.32 Ci/mmol, respectively.

binding patterns of BP or individual BP metabolites were examined by SDS-polyacrylamide gel electrophoresis and fluorography after incubation of microsomes with BP or metabolites and a NADPH-generating system (Fig. 3). In essence, appreciable binding occurred to a few proteins mainly in the MW region of 45,000–70,000. There

were differences, however, in the binding patterns of different metabolites, but the composite patterns essentially add up to the pattern of BP itself.

To facilitate a comparison, schematic binding patterns in the most interesting region are shown in Fig. 4. The major binding components have been assigned according

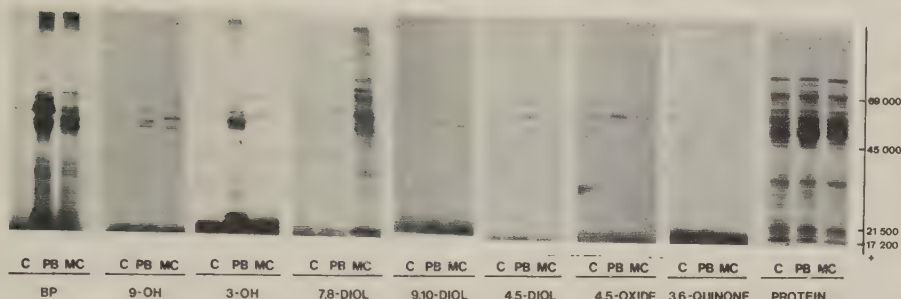


Fig. 3. Binding patterns of BP and BP metabolites to microsomal proteins from livers of control (C), phenobarbital (PB), and MC-pretreated rats.

Incubations were performed for 30 min at a substrate concentration of 13 μ M. The specific activities used were the same as in Fig. 1. Electrophoresis and fluorography were performed as described in the experimental section.

to their molecular weights. The most striking differences in binding specificity between microsomes from phenobarbital- and MC-treated rats occurred in two components, one with an apparent MW of 52,000 present in microsomes from phenobarbital-treated rats and one with a MW of 56,000 present in microsomes from MC-treated animals. These components had the same mobility in the gel as two prominent protein bands seen after phenobarbital or MC treatment, respectively. We have found in other experiments that these bands have the same mobilities as purified cytochromes *P*-450 and *P*-448. Another difference observed was in the binding to a 60,000 MW component, which was more prominent in microsomes from phenobarbital-treated rats than from MC-treated rats. The binding to control microsomes was comparatively weak for all substrates.

A comparison of the protein binding patterns of BP and BP metabolites to microsomes from phenobarbital-treated rats reveals that different metabolites yielded different patterns. The principal target for BP-4,5-dihydrodiol, BP-4,5-oxide, and BP was the 60,000 MW component. BP-7,8-dihydrodiol, on the other hand, bound equally well to this component and to the 50,000 MW component, while 3-OH-BP and 9-OH-BP bound more to the 50,000 and 52,000 MW components and less to that of 60,000 MW. There was also a strong radioactive

band at MW 20,000 in both the 3-OH-BP and the BP patterns (Fig. 3).

The protein binding pattern of BP-7,8-dihydrodiol to microsomes from MC-treated animals differed markedly from those of the other metabolites. There was little radioactivity associated with the otherwise conspicuous 56,000 MW component, and the pattern was dominated by the 50,000 MW component together with 58,000 and 72,000 MW bands that were specific for this metabolite. In contrast, BP, 3-OH-BP, and 9-OH-BP bound preferentially to the 56,000 MW component, but also to the 52,000 MW component. The intensity of the binding patterns obtained with the other metabolites was generally very weak (Fig. 3).

DISCUSSION

The results presented in this paper show both quantitative and qualitative differences in the irreversible binding to microsomal proteins of further activated BP metabolites. In general, the extent of binding was higher to microsomes from phenobarbital-treated and, particularly, from MC-treated animals than to microsomes from untreated animals. The requirement of a NADPH-generating system shows that metabolic activation is a prerequisite for significant binding to occur.

The metabolism of BP is enhanced in the 7,8 and 9,10 regions of the molecule when the reaction is catalyzed by microsomes from MC-treated rats, resulting in an increase in the formation of BP-7,8-dihydrodiol and 9-OH-BP (4). BP-7,8-dihydrodiol is further activated to the corresponding 9,10-oxide (7, 8, 12), whereas the active form of 9-OH-BP is probably the corresponding 4,5-oxide (12), both of which are major DNA binding species (12, 19, 20). We found here that BP-7,8-dihydrodiol bound more extensively to proteins in microsomes from MC-treated rats. The most likely ultimate binding species in this case was BP-7,8-dihydrodiol-9,10-oxide, in analogy with the activation preceding binding to DNA (9). It should be pointed out, however, that a final identification of binding metabolites has to await their isolation from microsomal proteins. The only BP



Fig. 4. Schematic protein binding patterns of BP and BP metabolites.

The electrophoretic patterns of Fig. 3 in the MW region 45,000-70,000 have been redrawn. A, BP; B, 9-OH-BP; C, 3-OH-BP; D, BP-7,8-dihydrodiol; E, BP-9,10-dihydrodiol; F, BP-4,5-dihydrodiol; G, BP-4,5-oxide; H, BP-3,6-quinone.

metabolite isolated after hydrolysis of microsomal proteins so far is BP-4,5-dihydrodiol, indicating that the binding agent in this case was BP-4,5-oxide (21) or a derivative of this oxide.

It is expected that BP-4,5-oxide should be one of the activated metabolites which bind irreversibly to microsomal proteins. However, the binding of BP-4,5-oxide was greatly enhanced in the presence of a NADPH-generating system, suggesting that an additional activation is required before binding. This indicates that the metabolic activation of BP starting with oxide formation in the 4,5 position might be more complicated than is presently recognized, and that several metabolic steps are required before binding to proteins.

The electrophoretic studies showed that the binding patterns to microsomal proteins differed both for the different metabolites studied and after various treatments of the animals. The binding pattern of BP to liver microsomes from phenobarbital-treated rats was dominated by a 60,000 MW band. Some of the BP metabolites examined, particularly BP-4,5-diol, BP-4,5-oxide, and BP-7,8-diol, also bound extensively to this component. However, there was a very low binding of BP and BP metabolites to the 60,000 MW component in microsomes from control or MC-treated animals. A possible explanation for the difference is that the component is induced by phenobarbital treatment. This appears less likely, however, since there is no visible enhancement in protein staining at 60,000 MW. We have also found in preliminary experiments that the binding of BP to 60,000 MW material in microsomes from control rats is enhanced after the addition of cytochrome P-450 purified from phenobarbital-treated rats.

An important question then is which factor(s) determines the specificity in the binding of BP metabolites to their target proteins. One possibility is that reactive metabolites will bind close to their site of activation in the membrane. This suggestion is borne out by the binding of metabolites to the 56,000 MW component in microsomes from MC-treated rats. This component comigrates with purified cytochrome P-448 in the gel, indicating that the binding protein is cytochrome P-448, an enzyme which participates in the metabolic activation of BP and its metabolites. Similar evidence applies to the 52,000 MW component in microsomes from phenobarbital-treated animals which comigrates with purified cytochrome P-450. We have also found in preliminary experiments that BP will bind irreversibly to a protein which comigrates with cytochrome P-450 on incubation with a reconstituted activating system consisting of NADPH-cytochrome P-450 reductase, cytochrome P-450, and NADPH. The identities of the metabolite binding components of 50,000 and 60,000 MW are not known presently, but we are examining whether the former might be epoxide hydratase.

The most likely metabolic route leading to covalent binding of metabolites to microsomal proteins is through the formation of reactive oxides by the mixed-function oxidase system. Provided that protein binding occurs close to the site of activation in the membrane, similar binding patterns would be expected for different BP metabolites after activation to oxides. The patterns dif-

fered markedly, however, and other additional explanations for the binding specificity have to be found. One possibility is that the kind of metabolite formed is crucial; important factors may be the positions of the reactive oxide or other substitutions in the molecule or the kind of primary metabolite that is activated (quinone, dihydrodiol, or phenol).

It is also possible that metabolic conversions other than oxide formation will produce reactive metabolites capable of binding covalently to proteins. For instance, dihydrodiols may be dehydrogenated to catechols with a subsequent formation of semiquinones or quinones. However, this pathway is catalyzed by dihydrodiol dehydrogenase, which is a soluble enzyme (22) and therefore most likely absent from our incubations.

It has been suggested that there is a connection between the binding of toxic compounds to proteins and their toxicity (10, 23). In order to evaluate this possibility intracellular target proteins for the toxic compounds should be isolated, and the effect of binding examined. So far, a few cytosolic proteins have been identified which bind polycyclic hydrocarbons irreversibly (24), but nothing is known of the identity of target proteins in other subcellular fractions. The present work shows that only a few microsomal proteins will bind metabolites of BP and that the protein binding patterns differ for the metabolites. Of prime importance now is to isolate microsomal target proteins and to examine in which manner the binding will affect their function.

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A RAPID AND SENSITIVE METHOD FOR DETERMINATION OF COVALENT BINDING OF BENZO[*a*]PYRENE TO PROTEINS

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SUMMARY

A method is presented for the quantitative determination of covalent binding of metabolically activated benzo[*a*]pyrene to microsomal proteins. After incubation of radiolabelled benzo[*a*]pyrene with microsomes and NADPH, the mixture is applied to filter paper discs. These are immersed in ethanol to precipitate the proteins. Unbound radiolabel is removed by repeated washes of the filters in organic solvents before scintillation counting. The method is simple, rapid, sensitive and accurate, and works both with ¹⁴C- and ³H-labelled compounds. The method is suitable for measuring the incorporation of other radiolabelled xenobiotics to proteins of both microsomes and other subcellular fractions and for the analysis of binding to isolated proteins.

INTRODUCTION

Many xenobiotics are known to bind covalently to cellular macromolecules, either directly or after metabolic activation by the microsomal mixed-function oxidase system [1,2]. The covalent binding of reactive metabolites to DNA and its possible correlation to mutagenesis and carcinogenesis have been extensively investigated [3]. In comparison, the binding of metabolites to proteins has received little attention, although quantitatively this binding exceeds that to DNA and RNA in several experimental systems [4,5]. The covalent binding of reactive metabolites of xenobiotics to proteins has been implicated in cell toxicity [5–8]. In this connection the findings that metabolically activated toxic and carcinogenic compounds will bind specifically rather than randomly to microsomal [9–11] or nuclear [12,13] proteins are of considerable interest.

Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital; SDS, sodium dodecyl-sulfate.

A serious limitation when studying the covalent binding of metabolically activated compounds to proteins is the lack of convenient quantitative analytical methods. Usually the incorporation is studied using radiolabelled substances. After the incubation unbound compounds have to be removed from the protein fraction containing radiolabelled adducts. This may be achieved through extractions with organic solvents and/or precipitation of the proteins and repeated washings of the precipitate [12–18]. Since each step involves a centrifugation the processing is rather cumbersome when several samples are to be analyzed. In addition, comparatively large incubations have to be performed with these methods in order to get an acceptable recovery of bound material. A recently described method [19] employs dialysis in the presence of SDS to remove unbound material. This method appears useful for measuring covalent binding to macromolecules in general, i.e., including nucleic acids and complex lipids, but it is not suitable for rapid analysis of the binding to proteins.

Using another method Uehleke et al. [20] have studied the covalent binding of ^{14}C -labelled carbon tetrachloride to microsomal proteins by precipitation of the proteins on filter paper discs after incubation. Trichloroacetic acid was employed for precipitation and washing of the filters. However, this technique is not suitable for analysis of the binding of polycyclic aromatic hydrocarbons since excess radiolabel can not be removed efficiently after precipitation in trichloroacetic acid.

In this report we present an alternative method for the quantitative analysis of benzo[*a*]pyrene covalently bound to proteins. It involves precipitation of proteins with bound radiolabelled benzo[*a*]pyrene metabolites on filter paper discs using organic solvents. Unbound radiolabel is then extracted with organic solvents while the proteins are still precipitated on the discs. The method is rapid, simple and sensitive, and it can easily be adapted for the analysis of other covalently bound xenobiotics. The incubation conditions for optimum binding have been studied.

MATERIALS AND METHODS

Chemicals

[^{14}C] Benzo[*a*]pyrene, [^3H] benzo[*a*]pyrene and [^{14}C] valine were obtained from the Radiochemical Centre, Amersham. The preparation of ^3H -labelled benzo[*a*]pyrene metabolites was as described earlier [21]. All chemicals were of analytical grade, except organic solvents used for washing of filters.

Preparation of microsomes

Liver microsomes were prepared from male Sprague–Dawley rats [10]. The animals had been treated with PB (0.1% in the drinking water for 5 days), MC (one i.p. injection of 80 mg/kg body wt. in corn oil 24 h before sacrifice) or were untreated (control microsomes). The specific contents of cytochrome *P*-450 determined according to Omura and Sato [22] were 1.43, 0.70 and 0.59 nmol/mg protein, respectively. Protein was determined by the Lowry method [23] with bovine serum albumin as standard.

Liver microsomes radiolabelled in the protein fraction were obtained in the following way. PB (75 mg/kg body wt.) was injected once i.p. followed after 4 and 18 h by two i.p. injections of [^{14}C]valine (10 μCi each; 285 mCi/mmol). The rats were killed 4 h after the last injection. Microsomes were prepared as above and washed twice more in buffer also containing 1 mM unlabelled valine. No free radiolabelled valine could be detected in this preparation by thin layer chromatography [24] and 99.8% of the radioactivity was recovered after precipitation with 5% trichloroacetic acid.

Analysis of the covalent binding of benzo[a]pyrene to microsomal proteins

The standard incubation procedure arrived at after optimization of the conditions was the following. The incubation mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 5 μM MnCl_2 , 2 mM NADPH, 25 μM [^{14}C]-benzo[a]pyrene (21.7 mCi/mmol) dissolved in 2.5 μl dimethylsulfoxide and 100 μg microsomal protein in a final volume of 100 μl . The incubation was started by the addition of benzo[a]pyrene. It was performed in stoppered tubes at 37°C for 30 min. The incubation was stopped by transferring a 50- μl aliquot to a filter paper disc (Whatman 3MM, diameter 2.5 cm) which was immediately immersed in 96% ethanol.

To remove unbound benzo[a]pyrene and benzo[a]pyrene metabolites formed during the incubation, the discs were washed consecutively in ethanol, twice in methanol, twice in acetone and once in *n*-hexane. Each wash was for 10 min with occasional agitations in 10 ml of solvent per disc. Several discs could be washed together without cross-contamination of radioactive material. The discs were dried and counted in 0.4% Omnifluor (New England Nuclear) in toluene using a Searle Mark III liquid scintillation counter. The recovery of radioactivity was 96% for ^{14}C and 53% for ^3H when compared to combusted samples. The values given have been compensated

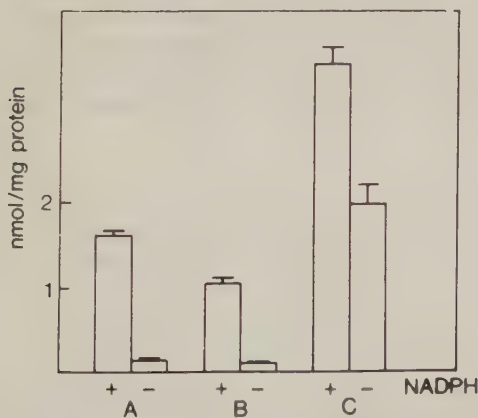


Fig. 1. A comparison between different extraction procedures. Scaled-up incubations were performed as described in Materials and Methods using microsomes (2 mg/ml) from MC-treated rats. Aliquots of 50 μl were applied on filter paper discs which were washed as described in Materials and Methods (A). Aliquots of 300 μl were extracted according to Tunek et al. [18] (B) or Jollow et al. [14] (C). Incubations without NADPH were performed as blanks. Each value represents the average of 10 determinations.

for recovery and counting efficiency. The standard deviation of the analyses performed with this method is 3.6% ($n = 10$) (cf. Fig. 1).

Analysis of the binding of benzo[a]pyrene to microsomal RNA

After incubation a scaled up standard incubation mixture (300 μ l) with microsomes from MC-treated rats was made 0.5 M in NaCl (final vol. 1 ml). The mixture was extracted twice in 1 ml of phenol previously saturated with water. Each of the phenol phases was washed with 0.5 ml of 0.5 M NaCl. The aqueous phases were combined and made 5% in SDS (final vol. 2 ml). Twelve millilitres of 95% ethanol saturated with sodium perchlorate was added and nucleic acids were allowed to precipitate overnight at -20°C . The precipitate was collected by centrifugation and washed 3 times with 70% ethanol. It was dissolved in 1 ml 0.15 M NaCl and 0.015 M sodium citrate (pH 7.2). RNA was determined with a modified orcinol method [25]. Bound benzo[a]pyrene was measured by scintillation counting in 5 ml Aquasol (New England Nuclear).

RESULTS

Precipitation of proteins and washing procedure

Two parameters are particularly important for a method to be useful in the analysis of protein-bound radiolabelled compounds. First, the recovery of radiolabelled protein adducts should be quantitative and, second, unbound material should be removed efficiently to keep the background low. Table I shows that prelabelled microsomal proteins are recovered quantitatively after precipitation on filter paper discs followed by washing according to our procedure. In contrast, earlier used methods [14,18] gave a slightly lower recovery of proteins.

The efficiency of the washing procedure was tested in experiments where radiolabelled benzo[a]pyrene or metabolites of benzo[a]pyrene were included in the incubation mixture without NADPH. Samples were applied on filters which were washed as in our method. The benzo[a]pyrene meta-

TABLE I

RECOVERY OF PROTEIN BY DIFFERENT EXTRACTION PROCEDURES

Microsomal protein (330 $\mu\text{g}/\text{sample}$) labelled with [^{14}C]valine was used. Each value represents the average of 10 measurements.

Treatment	Recovery	
	dpm	%
Before extraction	406 $\pm$ 16	100
Present method	407 $\pm$ 14	100
Tunek et al. [18]	330 $\pm$ 56	81
Jollow et al. [14]	299 $\pm$ 12	74

bolites tested (3-hydroxy, 9-hydroxy, 4,5-dihydrodiol, 7,8-dihydrodiol, 9,10-dihydrodiol and 4,5-oxide) were removed with a similar efficiency as benzo[*a*]pyrene itself. This indicates that metabolites formed during an incubation do not influence the background by not being removed efficiently during the washing procedure.

A comparison between different extraction methods after incubation of benzo[*a*]pyrene with microsomal proteins is presented in Fig. 1. Both the new procedure and the one we used earlier [18] gave low background values in the absence of NADPH. Unbound material was not extracted efficiently by a third method [14], which therefore yielded inexact results.

Incubation conditions

The time-dependent binding of benzo[*a*]pyrene to microsomal proteins is shown in Fig. 2. It was linear (microsomes from PB-treated or control rats) or close to linear (microsomes from MC-treated rats) for at least 60 min. The incorporation continued at a slightly slower rate after this time. At 220 min, 18, 9 and 6 nmol of metabolically activated benzo[*a*]pyrene or benzo[*a*]pyrene metabolites had bound per milligram microsomal protein from MC- or PB-treated and control rats, respectively. The incorporation in absence of NADPH was low indicating that metabolic activation was necessary for binding to occur. Despite the extensive binding to microsomes from MC-treated rats oxygen was not a limiting factor in our incubations.

The influence of the amount of microsomal protein on the incorporation of benzo[*a*]pyrene is seen in Fig. 3. The reaction was proportional to added

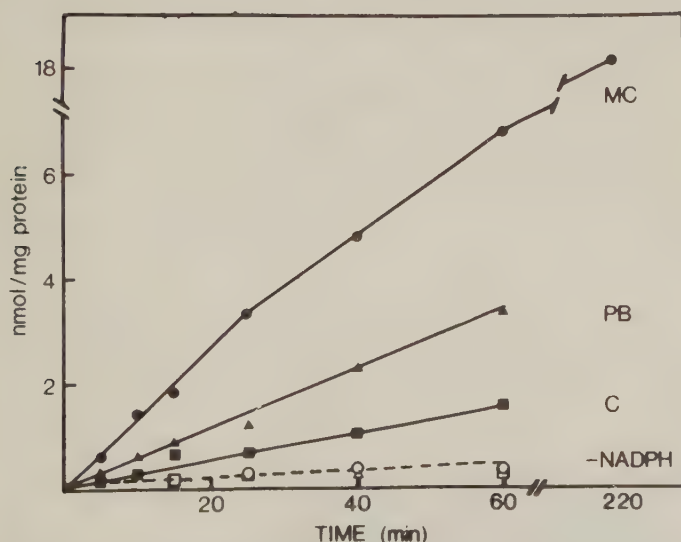


Fig. 2. Time-course of benzo[*a*]pyrene incorporation into microsomal proteins. Standard incubation conditions were used with liver microsomes from MC-treated (●), PB-treated (▲), and control (■) rats.

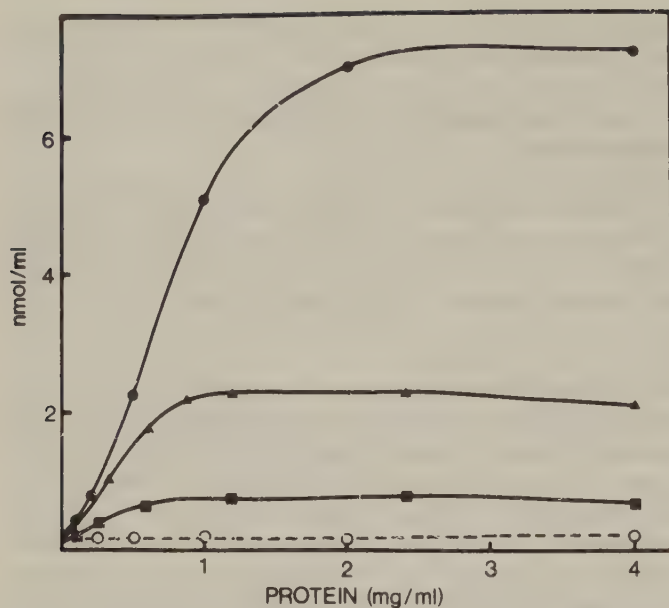


Fig. 3. The effect of protein concentration on benzo[a]pyrene incorporation. Standard incubation conditions with various concentrations of microsomal proteins from MC-treated (●), PB-treated (▲), and control (○) rats were used. Closed symbols are incubations with and open symbols without NADPH.

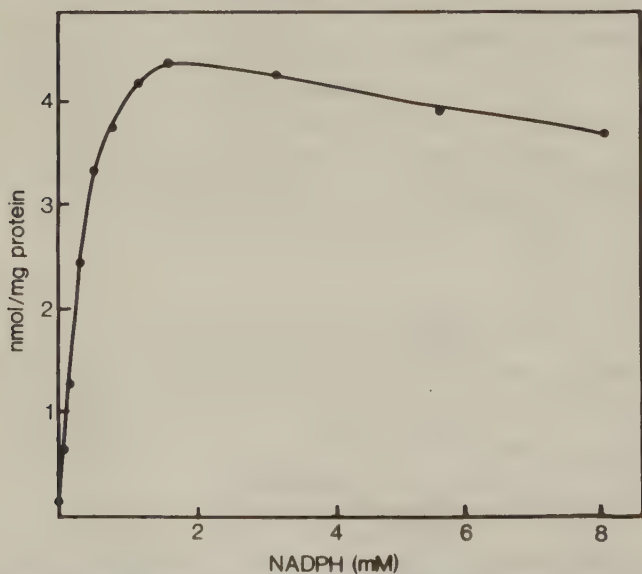


Fig. 4. The effect of NADPH on the incorporation of benzo[a]pyrene. Standard incubation conditions with liver microsomes from MC-treated rats and increasing concentrations of NADPH were used.

microsomal protein from MC-treated rats between 0.2–1 mg/ml incubation mixture. The incorporation was comparatively low below this interval, and was independent of added protein above 2 mg/ml. Similar curves were obtained with microsomes from control or PB-treated rats, although the incorporation was much lower in these cases.

The dependency on the concentration of NADPH for the incorporation of benzo[a]pyrene into microsomal proteins is shown in Fig. 4. The incorporation increased with increasing concentrations of NADPH reaching a maximum at 1.6 mM. A slight inhibition of the reaction was observed at higher concentrations. As an alternative we tested the effect of a NADPH-generating system [18]. Then the incorporation was approx. 30% of that obtained with an optimal concentration of NADPH. Obviously the system did not generate a sufficient steady-state concentration of NADPH, although the concentration of NADP was 0.85 mM and most of this is supposed to be kept in the reduced state during the incubation.

The influence of the benzo[a]pyrene concentration on the extent of incorporation was also examined (Fig. 5). A maximum binding was obtained at a concentration of 25 μ M. This concentration is similar to that yielding a maximum incorporation of benzo[a]pyrene into nuclear proteins [17], but is only half of that yielding a maximum benzo[a]pyrene mono-oxygenase activity [26].

Incorporation into microsomal RNA

The possibility that benzo[a]pyrene or its metabolites was incorporated into microsomal RNA rather than into proteins was also considered. The specific binding of benzo[a]pyrene was 31 pmol/mg RNA in a standard

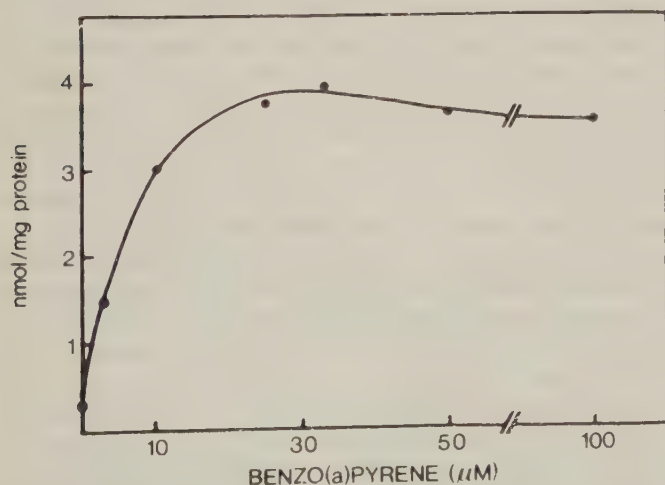


Fig. 5. The effect of benzo[a]pyrene concentration on its incorporation into microsomal proteins. Standard incubation conditions with liver microsomes from MC-treated rats and increasing concentrations of benzo[a]pyrene were used.

incubation as compared to 4000 pmol/mg microsomal proteins. Since the ratio of protein to RNA in these microsomes was 7 : 1 (w/w), only 0.1% of the benzo[*a*]pyrene that bound to macromolecules was incorporated into RNA under our incubation conditions.

DISCUSSION

The presented method allows a simple, rapid, sensitive and accurate quantitative analysis of the covalent binding of xenobiotics to proteins. The binding of benzo[*a*]pyrene and its metabolites to microsomal proteins has been examined here, but the method can be extended to measure the covalent binding of other radiolabelled xenobiotics to proteins from various subcellular fractions provided that unbound radiolabelled substances can be removed from the filter paper discs without dissolving the protein fraction. For instance, we have used the method to study the incorporation of metabolically activated benzo[*a*]pyrene to cytosolic proteins (Schelin et al., in prep.). The method can be used both for ^3H -labelled and ^{14}C -labelled compounds, although the counting recovery of ^3H is much lower than of ^{14}C (53% and 96%, respectively, in our scintillation counter), as is the counting efficiency.

The analysis of protein-bound chloroform after precipitation with trichloroacetic acid on filter papers or glassfibre-filters followed by trichloroacetic acid and alcohol washes has been described [20,27]. This precipitation and washing procedure was not efficient, however, for a polycyclic aromatic hydrocarbon as benzo[*a*]pyrene.

An advantage with our method is its sensitivity. The incorporation of benzo[*a*]pyrene to microsomal proteins from MC-treated rats could be measured accurately using 10 μg of microsomal protein (0.1 mg/ml in the incubation mixture). In comparison, several mg of microsomal proteins have been used in other studies [11,15,16], presumably due to the requirement of bulk proteins for a good recovery during the extraction procedure. This is not a problem when using the present method since the proteins are kept precipitated on the filters during the entire washing. Since the incorporation of benzo[*a*]pyrene (and presumably also of other xenobiotics) is not proportional to the concentration of microsomal proteins above 1–2 mg/ml (which is a much lower protein concentration than what has been used in other binding studies) this new method should allow a more accurate analysis of the specific incorporation of xenobiotics into proteins.

The washing procedure of the filters may be simplified by omitting one or more of the steps. This tends, however, to give a higher background and less reproducible results. If the incorporation is considerably higher than the background this may not be a serious drawback. We have found, for instance, that one methanol and the *n*-hexane washing step can be omitted if maximum accuracy is not required. When the incorporation of other xenobiotics than benzo[*a*]pyrene is examined, changes in the washing procedure may be necessary. For instance, polar substances may need more extensive washing in polar solvents and non-polar ones in non-polar solvents.

As the precipitation and washing procedure is outlined here a quantitative recovery of DNA and RNA is also obtained. It should be possible to extract the nucleic acids from the filters by altering the procedure. This should be of interest when the binding of xenobiotics to nuclear components is examined.

The covalent nature of the binding between radiolabelled benzo[a]pyrene and proteins is suggested by several pieces of evidence. The bound radio-label cannot be removed from the filter paper discs by organic solvents or other protein precipitants we have tested, such as trichloroacetic acid. This, together with the metabolic activation required for binding and the efficient extraction of both benzo[a]pyrene and isolated benzo[a]pyrene metabolites from the filter papers, precludes even a high-affinity non-covalent binding between benzo[a]pyrene and the precipitated proteins (or the filters). We also know from our earlier experiments [9,10] that both benzo[a]pyrene and metabolites of benzo[a]pyrene will migrate with protein fractions on electrophoresis after denaturation with SDS.

The binding of benzo[a]pyrene to microsomal proteins under our incubation conditions is rather extensive. The maximum binding we have observed, close to 20 nmol bound per milligram protein, corresponds to a unity molar ratio between incorporated benzo[a]pyrene and microsomal proteins, assuming an average molecular weight of 50 000 for the proteins. Then approx. 70% of the added benzo[a]pyrene had bound to proteins, either after one or more cycles of metabolic activation (cf. Ref. 10). Studies of the binding of other xenobiotics to proteins after incubations with microsomes show similar high levels of incorporation using other methods of analysis [11,15,19].

In summary, the presented method allows an accurate analysis of the incorporation of small amounts of metabolically activated xenobiotics to proteins of various subcellular fractions. Because of its sensitivity the method should be useful in studies on the incorporation of xenobiotics to specific target proteins, whether isolated or in microsomes or other subcellular fractions.

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COVALENT BINDING OF BENZO[a]PYRENE TO RAT LIVER CYTOSOLIC PROTEINS AND ITS EFFECT ON THE BINDING TO MICROSOMAL PROTEINS

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Abstract—Benzo[a]pyrene will bind covalently to rat liver cytosolic proteins when incubated with microsomes and NADPH. The binding is most extensive when microsomes from 3-methylcholanthrene-treated rather than phenobarbital-treated or control rats are used. The binding to cytosolic proteins increases when incubations are performed with increasing concentrations of cytosol. At the same time the covalent binding of benzo[a]pyrene to microsomal proteins decreases. Two cytosolic polypeptides are the main targets for benzo[a]pyrene. These have the same mobility in polyacrylamide gels as the subunits of purified glutathione S-transferase B. These subunits also react covalently with benzo[a]pyrene when the transferase is incubated with microsomes and NADPH.

Several xenobiotics are converted to chemically reactive metabolites by the microsomal cytochrome P-450-linked monooxygenase system. These metabolites may bind covalently to tissue macromolecules [1-4] thereby eliciting their harmful effects. Covalent binding of xenobiotics to microsomal [3, 5], nuclear [4, 6, 7] and cytosolic [8-11] proteins has been reported. Recent evidence indicates that such binding occurs preferentially to some proteins [6, 12-15] rather than being a random event. Thus, ligandin is a preferred target in rat liver cytosol for polycyclic aromatic hydrocarbons administered *in vivo* [4, 8, 11]. Both 3-MC† and B[a]P will bind covalently to soluble proteins *in vitro* when incubated with postmitochondrial supernatant and reduced pyridine nucleotides [8, 10]. Therefore, metabolic activation of these compounds, presumably catalyzed by microsomal enzymes, seems to be required for binding.

In this report is shown that irreversible binding of B[a]P to cytosolic proteins is dependent on microsomes. It is also shown that the binding to cytosolic proteins results in a corresponding decrease in binding to microsomal proteins. This observation might be of importance in the assessment of the toxicity of polycyclic aromatic hydrocarbons and other compounds that undergo metabolic activation.

EXPERIMENTAL

Chemicals. [7,10-¹⁴C]B[a]P (21.7 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. Different forms of glutathione S-transferase (EC 2.5.1.18) purified according to [17] were a generous gift from Dr. B. Ketterer, London.

Animals and treatments. Male Sprague-Dawley

rats (Anticimex, Stockholm) weighing 150-200 g, were allowed free access to food and water. The animals were treated with PB (0.1% in the drinking water for 5 days), 3-MC (one intraperitoneal injection of 80 mg/kg body weight in corn oil 24 hr before sacrifice), or were untreated (control animals).

Preparation of microsomes and cytosol. Microsomes were prepared as described earlier [12]. The cytosol was obtained as the 105,000 g supernatant, and was chromatographed through Sephadex G-25 (3 × 20 cm column) to remove low molecular weight material. The column was previously equilibrated and developed in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 25 mM KCl. The material eluting with the void volume was collected. Both microsomes and cytosol were stored in aliquots at -20°.

Analytical procedures. Cytochrome P-450 was determined according to Omura and Sato [18]. The average cytochrome P-450 content was 0.60, 2.17 and 1.10 nmol/mg protein in microsomes from control, PB- and 3-MC-treated animals, respectively. Protein was determined by the Lowry procedure [19] using bovine serum albumin as standard.

Quantitative determination of covalently bound B[a]P. The binding of B[a]P to microsomal and cytosolic proteins was analysed as described in detail by Wallin *et al.* [20]. The incubations contained 10 mM Tris-HCl, pH 7.4, sample (0.20 mg microsomal and/or 0.72 mg cytosolic protein), 2 mM NADPH and B[a]P (added in 3 µl DMSO) in a volume of 200 µl. After incubation the proteins were precipitated on filterpaper discs and unbound B[a]P was extracted as described [20]. The incorporated radiolabel was measured in 0.4% Omnifluor (New-England Nuclear) in toluene. In experiments where microsomes and cytosol were separated after the incubation, the volume was scaled-up to 2.5 ml. These latter incubations were for 30 min and the samples were centrifuged at 105,000 g for 1 hr at 4°. The resulting microsomal pellet was suspended in

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‡ Abbreviations: B[a]P, benzo[a]pyrene; 3-MC, 3-methylcholanthrene; PB, phenobarbital.

1 ml of 50 mM Tris-HCl, pH 7.4, using a Potter homogenizer.

Analysis of protein binding patterns. Samples containing 70 μ g of protein obtained from the incubations were treated for SDS-polyacrylamide gel electrophoresis and electrophoresed in slab gels as described earlier [21, 22]. After staining and destaining gels were treated with 2,5-diphenyl-oxazole and fluorographed [23] using Kodak X-Omat films.

RESULTS AND DISCUSSION

The covalent binding of B[a]P to rat liver cytosolic and microsomal components was examined first. In the presence of NADPH [14 C]B[a]P became bound irreversibly to microsomal proteins. This reaction was linear with time for around 20 min and reached a maximum after 30 min (Fig. 1). The rate of covalent binding (and also the maximum binding) was much higher into microsomes from 3-MC-treated rats than into microsomes from PB-treated or control rats (initial rates were 150, 50 and 15 pmol/min per mg protein, respectively). No significant binding occurred in the absence of NADPH indicating that a metabolic activation of B[a]P preceded binding.

The possibility that B[a]P would form adducts with cytosolic proteins upon incubation with cytosol and NADPH was then examined (Fig. 1). The specific

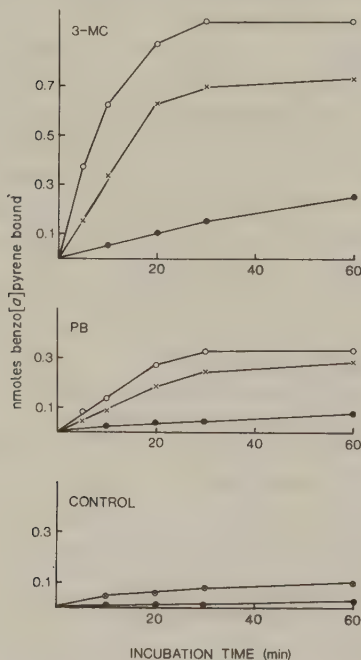


Fig. 1. Time courses of B[a]P binding to microsomal and cytosolic proteins. Microsomes (x), cytosol (●), or a microsome-cytosol mixture (○) were incubated and the binding analysed as described in Experimental. Liver microsomes and cytosol were in each case from control, 3-MC- or PB-treated rats.

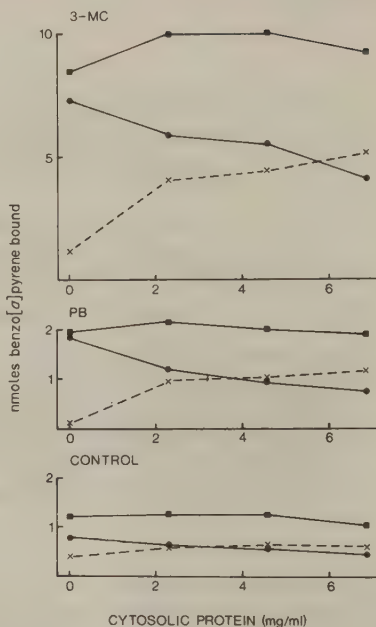


Fig. 2. The binding of B[a]P to soluble and microsomal proteins after incubation with increasing amounts of cytosol. Incubations were as described in Experimental. The soluble fraction (x) and microsomes (●) were separated by centrifugation after the incubation. The total binding (■) is also indicated.

irreversible binding observed with cytosol was approximately 20 times lower than with microsomes (note that 0.72 mg of cytosolic protein was used in the incubations compared to 0.20 mg of microsomal protein). α -Naphthoflavone, SKF 525A or CO inhibited this binding indicating that it was due to a slight microsomal contamination rather than to an intrinsic activity of the cytosol.

To examine whether microsomal enzymes would catalyse a binding of B[a]P into cytosolic components, these fractions were combined and incubated with NADPH (Fig. 1). The initial rate of B[a]P binding into the combined fractions isolated from 3-MC-treated rats was 50–100% higher than expected from the sum of the individual fractions. No such enhancement was seen in the combined fractions of PB-treated or control rats.

Since these experiments did not give clear evidence of a microsome-catalysed binding of B[a]P to cytosolic components, the matter was examined further by incubating combined fractions with B[a]P and NADPH, and separating the fractions by centrifugation before quantification of the binding (Fig. 2). It was then found that a substantial amount of B[a]P reacted covalently with the soluble fraction. Furthermore, if the amount of cytosol in the incubation mixture was increased relative to microsomes a progressively larger part of B[a]P became bound to the

(a)

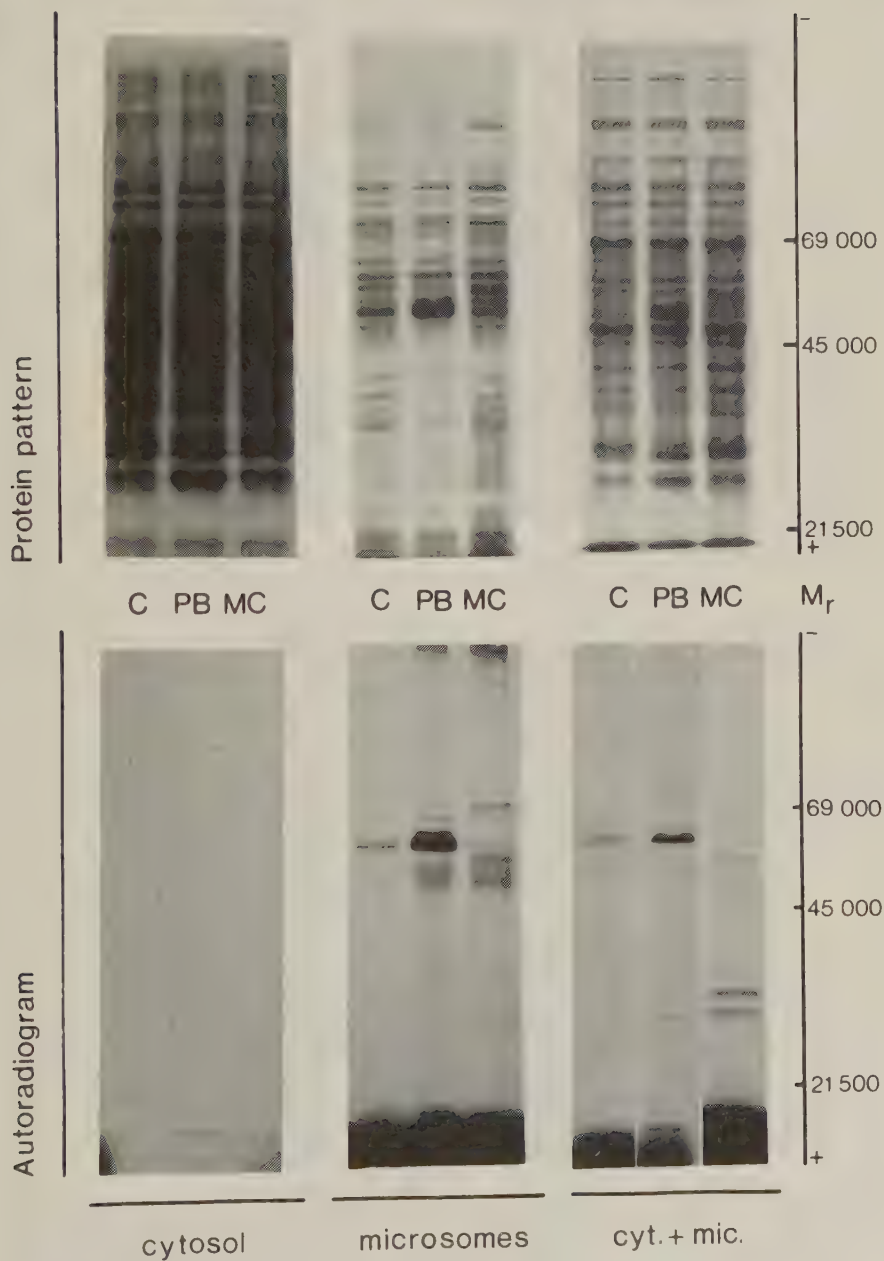


Fig. 3(a). Legend on p. 1504.

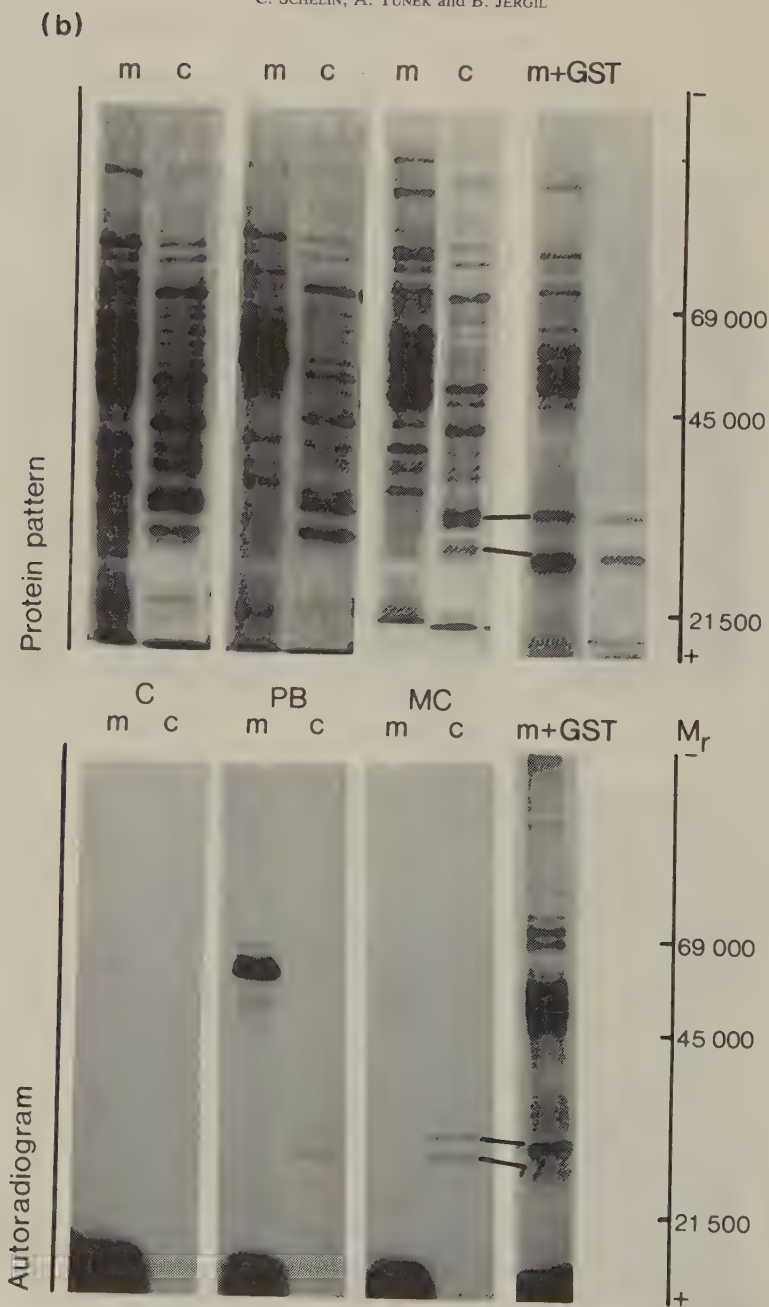


Fig. 3. B[a]P binding patterns. Samples prepared from control (C), PB- or 3-MC-treated rats were incubated with [14 C]B[a]P and NADPH and electrophoresed in SDS-polyacrylamide gels before fluorography (a), protein patterns and fluorographs of cytosol, microsome (m) and cytosol-microsome mixtures (b), protein patterns and fluorographs of microsomes (m) and cytosol (c) separated by centrifugation after incubation together. Microsomes from 3-MC-treated rats were also incubated with purified glutathione *S*-transferase B (m + GST). These latter patterns were obtained from electrophoretic runs with different separation in the low molecular weight region as indicated.

soluble fraction, while the binding to microsomes decreased correspondingly keeping the total binding constant. The binding to cytosolic components even exceeded that to microsomal components at high cytosol concentrations. A similar redistribution of bound B[a]P was observed regardless of whether the subcellular fractions were obtained from 3-MC- or PB-treated, or from control rats. The conclusion to be drawn is that microsomal enzymes catalyse a metabolic activation of B[a]P that leads to a covalent binding to soluble components. There also seems to be a competition between the microsomal and soluble fraction for the activated B[a]P.

The target specificity of the bound B[a]P was examined by electrophoresis in SDS-polyacrylamide gels and fluorography. As expected, no radioactive bands were detected in samples when cytosol alone had been incubated with radio-labelled B[a]P and NADPH (Fig. 3a). The patterns obtained with microsomes showed radiolabelled bands in the 50–70,000 M_r region. A dominating band ($M_r = 60,000$) was seen in PB-treated and control microsomes, while the binding was more equally distributed between several components in microsomes from 3-MC-treated animals. These patterns have been discussed in detail elsewhere [12, 13]. After incubation of cytosol and microsomes together two additional radiolabelled components (M_r 28,000 and 30,000) were evident when the subcellular fractions had been isolated from PB- or 3-MC-treated rats (Fig. 3b). Corresponding bands, although very faint, were seen in incubations from control animals. Further evidence that these additional bands belonged to the soluble fraction was obtained after centrifugation of the cytosol-microsome incubation mixture followed by electrophoresis and fluorography. Both these components remained in the supernatant, while the microsomal pellets yielded radiolabelled patterns similar to those obtained after incubation with microsomes alone. No other radiolabelled bands were detected in the supernatant.

The two soluble components that became modified through reaction with B[a]P could be identified as the subunits of glutathione *S*-transferase B (ligandin). When purified transferase was incubated with B[a]P, NADPH and liver microsomes, B[a]P was bound to two components with the same mobility in SDS-polyacrylamide gels as the soluble components of the above experiments and as the purified transferase (Fig. 3).

Our results thus confirm and extend the recent finding that B[a]P forms adducts with glutathione *S*-transferase B when incubated with postmitochondrial supernatant and NADPH [10]. In contrast to this work we found that both subunits of the transferase bound B[a]P. On the other hand, we did not observe the other binding components reported there. It is not clear whether these differences are due to different incubation conditions in the two cases.

The extent of B[a]P binding to cytosolic components can be calculated from the data of Fig. 2. Thus, approximately 0.7 nmoles bound per mg of cytosolic protein in the presence of liver microsomes from 3-MC-treated rats. Assuming that glutathione *S*-transferase B represents 2% of the total cytosolic

protein, this binding is close to a one to one ratio between B[a]P and transferase subunit. This is a considerably lower value than the probably incorrect binding capacity reported in [10] (1 mole/mg of partly purified glutathione *S*-transferase B, i.e. close to one B[a]P incorporated per amino acid residue when corrected for transferase purity).

So far it is not known which reactive metabolite(s) of B[a]P will bind to the cytosolic proteins. Microsomal enzymes from both PB- and 3-MC-pretreated animals will catalyze the binding even though they yield different metabolites [24]. Thus both K- and bay-region epoxides as well as secondary dihydrodiol epoxides might be implicated in the covalent binding. We have shown earlier that metabolically activated B[a]P-7,8-dihydrodiol will bind more efficiently than other B[a]P metabolites to microsomal proteins [13].

The physiological consequences of the covalent binding of B[a]P to glutathione *S*-transferase B (or any other target protein in the cell) is still uncertain. A decline in the catalytic function of the enzyme on binding was observed in [10], but there is no information on how its ability to bind substances non-covalently is affected. One interesting observation, however, is the decrease in covalent binding of B[a]P to microsomal proteins found here in the presence of cytosol. The physiological significance of this competition between microsomal and cytosolic proteins for B[a]P metabolites is still a matter for speculation. It has been shown, however, that glutathione and glutathione *S*-transferase B will inhibit the incorporation of B[a]P and B[a]P metabolites into DNA of isolated hepatic nuclei [25].

In summary, B[a]P binds covalently to rat liver cytosolic proteins after metabolic activation in the presence of microsomes and NADPH. The two subunits of glutathione *S*-transferase B are the preferred targets. The binding of B[a]P into cytosolic proteins leads to a corresponding decrease in binding into microsomal proteins.

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COVALENT BINDING OF BENZO(a)PYRENE TO CYTOCHROME P-450_{βNF-B2} AND
OTHER PROTEINS IN RECONSTITUTED MIXED-FUNCTION OXIDASE SYSTEMS.

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Abbreviations: SDS, sodium dodecylsulfate;
HPLC, high-pressure liquid chromatography;
cytochrome P-450_{βNF-B2}, the major form induced by
β-naphtoflavone;
cytochrome P-450_{pB-B2}, the major form induced by
phenobarbital;

ABSTRACT

A reconstituted mixed-function oxidase system, containing the major β -naphtho-flavone-induced isozyme of rat liver cytochrome P-450 bound benzo(a)pyrene covalently in the presence of NADPH. NADPH-cytochrome P-450 reductase was required for binding, and a maximum rate of adduct formation was obtained at 8 U of reductase per nmol cytochrome P-450. Phosphatidylcholine inhibited this reaction. Benzo(a)pyrene was bound to the cytochrome, but not to the reductase, as shown by SDS-polyacrylamide gel electrophoresis. Approximately 6 molecules of benzo(a)pyrene bound to each molecule cytochrome P-450 during prolonged incubations. No binding occurred when the β -naphthoflavone-induced isozyme of cytochrome P-450 was replaced by the major isozyme induced by phenobarbital, but both cytochromes incorporated benzo(a)pyrene to approximately the same extent when they were incubated together in the presence of the reductase and NADPH. Metabolically activated benzo(a)pyrene also bound covalently to purified epoxide hydrolase, when this enzyme was added to the reconstituted mixed-function oxidase system.

INTRODUCTION

Many carcinogenic and mutagenic polycyclic aromatic hydrocarbons, including benzo(a)pyrene, are converted to electrophilic metabolites by the microsomal mixed-function oxidase system. Most of these metabolites are converted further to more polar species that can be excreted readily from the cell (1,2). The reactive metabolites may also bind covalently to cellular macromolecules (1-4). This binding has been implicated in the toxic, mutagenic and carcinogenic properties of these compounds (5-9). The binding of metabolites to DNA has been studied extensively due to its possible connection with carcinogenicity (2,4,10). The binding to proteins, on the other hand, which in the case of polycyclic aromatic hydrocarbons by far exceeds the binding to DNA or RNA in various cells (7,11), has not been examined in detail. As a consequence, the physiological significance of the binding to proteins is less well known, although it has been implicated in cell toxicity (3,5,7,8).

It is now established that after metabolic activation polycyclic aromatic hydrocarbons and other toxic substances bind specifically rather than randomly to proteins in various subcellular fractions. Thus, histones have been identified as target proteins in the nucleus (3,7,12) and glutathione transferase B. (ligandin) in the cytosol (3,13-16). As for microsomes, i.e. the site of metabolic activation of these compounds, little is known about the identity of the target proteins. Enzymes in the mixed-function oxidase system have been suggested to bind xenobiotics covalently (17-22). Chloramphenicol and parathion have been

shown to form adducts with cytochrome P-450 after metabolic activation by a reconstituted mixed-function oxidase system (23,24) and this binding causes an inactivation of the cytochrome.

In order to identify target proteins for benzo(a)pyrene, we have incubated this hydrocarbon with purified microsomal enzymes which are part of the mixed-function oxidase system. We present evidence that cytochromes P-450 as well as epoxide hydrolase will bind metabolically activated benzo(a)pyrene.

MATERIALS AND METHODS

Chemicals.

(¹⁴C)-benzo(a)pyrene (21.7 mCi/nmole) was obtained from the Radiochemical Centre, Amersham. 2',5'-ADP-Sepharose 4B was from Pharmacia Fine Chemicals, L- α -phosphatidylcholine type V-E from Sigma, Renex 690 from Kemi-Intressen, Sundbyberg, Sweden, and Omnifluor and Enlightning from New England Nuclear. All other chemical used were of analytical grade and purchased from common commercial sources.

Preparation of microsomes.

Male Sprague-Dawley rats (Anticimex, Stockholm) weighing 150-200 g were given β -naphthoflavone (40 mg/kg) in 0.5 ml corn oil intraperitoneally for 3 days before sacrifice, or phenobarbital (0.1 % in the drinking water) for 5 days before sacrifice. Microsomes were prepared as described earlier (23).

Purification of NADPH-cytochrome P-450 reductase.

The enzyme was isolated from rats pretreated with phenobarbital using the following modifications of the method of Yasukochi and Masters (25). One volume of microsomal suspension was solubilized in 3 volumes of 25 mM Tris-HCl, pH 7.7, 10 mM EDTA, 0.1 mM dithioerythritol, 0.5 % sodium cholate, and 1.6 % Renex 690. After centrifugation at 105 000 g for 90 min, the solubilized material was applied directly to a 2',5'-ADP-Sepharose 4B column previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.7, 0.4 mM EDTA, 0.1 mM dithioerythritol, 0.1 % Renex 690, and 24 % glycerol. After washing the column the reductase was eluted with 1 mM 2'-AMP in the same buffer mixture. Fractions containing reductase were combined and treated with charcoal, SiO₂ (diatomaceous earth), and Amberlite XAD-2 to remove 2'-AMP and detergents. The final preparation had a specific activity of 25 units of NADPH-cytochrome P-450 reductase per mg protein. The enzyme was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. One unit is defined as the amount of enzyme catalyzing the reduction of 1 μ mol

cytochrome c per min at 22°C.

Purification of cytochrome P-450 and epoxide hydrolase.

Epoxide hydrolase and the major forms of cytochrome P-450 from liver microsomes of phenobarbital-(cytochrome P-450_{PB-B2}) and β -naphthoflavone-(cytochrome P-450 _{β NF-B2}) treated rats were purified as described by Guengerich and Martin (26) with the following modifications: After sample application the octylamino-Sepharose column was washed with buffer containing 0.5 % rather than 0.42 % sodium cholate. The P-450 was then eluted with buffer containing 0.08 % rather than 0.06 % Lubrol PX and 0.40 % rather than 0.33 % sodium cholate. The cytochrome P-450 fraction from octylamino-Sepharose was chromatographed on a 2 x 90 cm column of DEAE-Sephacel. A 1.5 liter linear gradient of 25 mM to 100 mM NaCl in buffer was used to elute the various P-450 forms. The altered conditions were necessary to separate the major P-450 fraction (B2) from a later fraction of slightly higher molecular weight (B3) present in the microsomes from both the phenobarbital- and β -naphthoflavone-treated rats.

The B2 fractions and the epoxide hydrolase fraction II used in this study were >95 % pure as judged by SDS-polyacrylamide gel electrophoresis (cf fig 5). The specific heme content of these cytochrome P-450 preparations was 15 nmol/mg protein. The specific activity of the epoxide hydrolase used was 215 nmol styrene glycol hydrolysed per min and mg protein (27).

Quantitative determination of covalently bound benzo(a)pyrene.

The covalent binding of benzo(a)pyrene to proteins was analyzed as described in detail by Wallin et al. (28). The incubation mixtures contained in a final volume of 200 μ l, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2 mM NADPH, and 25 μ M benzo(a)pyrene. Cytochrome P-450_{PB-B2} or P-450 _{β NF-B2}, NADPH-cytochrome P-450 reductase, epoxide hydrolase and phosphatidylcholine were added as indicated in each case. The incubation time was 30 min, unless otherwise indicated. After incubation the proteins were precipitated on filter paper discs and unbound benzo(a)pyrene was extracted before analysis by scintillation counting. Some incubations were performed in the presence of 0.1 mM α -naphthoflavone, 0.1 mM metyrapone, or 2 mM NADH.

Analysis of protein-binding patterns by electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (29) using 8 % slab gels. The incubation mixtures had the same composition as for quantitative analyses described above. In addition, 0.333 U NADPH-cytochrome

P-450 reductase, 0.025 nmol cytochrome P-450, 0.125 nmol epoxide hydrolase, or 50 μ g microsomal proteins was added as indicated in each case. After incubation for 30 min an equal volume of denaturation mixture (29) was added and the samples were boiled for one min. After electrophoresis gels were stained over night in 0.1 % Coomassie Brilliant Blue dissolved in 50 % methanol and 7 % acetic acid. After destaining in 25 % methanol and 7 % acetic acid the gels were soaked in 5 volumes of Enlightning for 30 min. The gels were dried under vacuum with heat, and exposed to Kodak X-Omat films at -70°C for 3 weeks. Molecular weight standards used were bovine serum albumin ($M_r = 69,000$), ovalbumin ($M_r = 45,000$) and carbonic anhydrase ($M_r = 30,000$).

Analytical procedures.

Protein was determined by the Lowry method (30) using bovine serum albumin as standard. Cytochrome P-450 was determined according to Omura and Sato (31), and NADPH-cytochrome P-450 reductase (using cytochrome c as substrate) according to Hrycay and O'Brien (32). The analyses were performed in duplicate.

RESULTS

Binding of benzo(a)pyrene to mixed-function oxidase components.

The possibility that benzo(a)pyrene would bind covalently to the components of a reconstituted mixed-function oxidase system consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidylcholine was examined first (Fig. 1). Binding, increasing linearly with time for approximately 30 min, occurred in the system containing cytochrome P-450_{BNF-B2}. The binding was dependent on added NADPH, indicating a requirement for metabolic activation of the benzo(a)pyrene. In contrast, there was no binding to components in a system containing cytochrome P-450_{PB-B2}.

Effect of NADPH-cytochrome P-450 reductase.

The effect of the amount of NADPH-cytochrome P-450 reductase on the covalent binding of benzo(a)pyrene in the reconstituted system is shown in Fig. 2. No binding occurred in the absence of reductase, but binding increased with increasing concentrations of the enzyme reaching an optimum at approximately 0.4 U of reductase added (8 U per nmole cytochrome P-450).

Effect of phosphatidylcholine.

The metabolism of a number of substrates including benzo(a)pyrene (33,34) in reconstituted mixed-function oxidase systems is dependent on added phospholipids.

The effect of phosphatidylcholine on the covalent binding of benzo(a)pyrene to components of reconstituted systems was therefore examined (Fig. 3). The highest binding was obtained when no phospholipid was added, whereas the binding was inhibited up to 50 % by increasing concentrations of phosphatidylcholine.

Cytochrome P-450 concentration.

The effect of the amount of cytochrome P-450 on the rate of benzo(a)pyrene binding in the reconstituted system was examined using a fixed molar ratio of reductase to cytochrome (Fig. 4). The initial rate of binding was proportional to the amount of cytochrome P-450_{βNF-B2} added up to 0.05 nmol. When this isozyme was replaced by cytochrome P-450_{PB-B2} the binding was negligible even at high concentrations of enzyme, thus corroborating the results of Fig. 1.

Effect of NADH and inhibitors of cytochrome P-450 activity.

When NADPH was replaced by NADH in the incubation mixture, the binding of benzo(a)pyrene decreased by more than 90 % (Table 1). A similar low degree of binding was observed in the presence of α-naphtoflavone, an inhibitor of the 3-methylcholanthrene-induced form of cytochrome P-450 (35). Metyrapone, an inhibitor of the phenobarbital-induced form of cytochrome P-450 (36), also inhibited benzo(a)pyrene binding in the reconstituted system containing cytochrome P-450_{βNF-B2}, but to a somewhat smaller extent than α-naphtoflavone did.

Protein targets for benzo(a)pyrene.

The identity of the protein targets for the metabolically activated benzo(a)-pyrene was examined by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 5). When a reconstituted system was incubated with NADPH the covalent binding of radiolabelled benzo(a)pyrene occurred to cytochrome P-450_{βNF-B2}, whereas there was no binding in the position of NADPH-cytochrome P-450 reductase (lane e). As expected, cytochrome P-450_{PB-B2} did not form adducts with benzo(a)-pyrene when incubated in place of P-450_{βNF-B2} in the reconstituted system (lane d). When the cytochromes were incubated together, however, binding occurred to both of them (lane f), and interestingly enough both cytochromes bound benzo(a)pyrene to approximately the same extent. This shows that target groups are present in cytochrome P-450_{PB-B2}, but that this cytochrome does not catalyze the formation of benzo(a)pyrene metabolites able to react with these groups. No radioactivity was present in the position of NADPH-cytochrome P-450 reductase in any of the incubations, yet this enzyme had to be present for the formation of protein adducts (lanes b and c).

The possibility that epoxide hydrolase could be a target for activated benzo(a)pyrene was also examined. Covalent binding to this enzyme was observed in the presence of cytochrome P-450_{BNF-B2} and reductase (lane g). The extent of binding to epoxide hydrolase was, however, less than that to the cytochrome.

As a comparison with the reconstituted systems, the binding of benzo(a)pyrene to components of liver microsomes from control, phenobarbital- and 3-methylcholanthrene-pretreated rats is also shown (lanes i-k). A radiolabelled band is seen in the position of cytochrome P-450_{BNF-B2} in the microsomes from β -naphthoflavone-pretreated animals, together with a band in the position of epoxide hydrolase and a strong intermediate band. Benzo(a)pyrene bound predominantly to one component in liver microsomes from phenobarbital-pretreated rats. This component had a higher molecular weight than cytochrome P-450_{PB-B2} and did not correspond to any labelled component in microsomes from 3-methylcholanthrene-pretreated rats. These radiolabelling patterns verify our earlier observations (17).

A rather strong and diffuse zone of radiolabel was observed at the top of the fluorographs. Since there was no corresponding protein staining, this was probably due to benzo(a)pyrene or uncharged benzo(a)pyrene metabolites not removed in the electrophoresis procedure.

DISCUSSION

We have shown here that cytochrome P-450_{BNF-B2} in the presence of NADPH-cytochrome P-450 reductase and NADPH will bind benzo(a)pyrene covalently, whereas the reductase is not a target for the aromatic hydrocarbon. We have also shown that epoxide hydrolase and cytochrome P-450_{PB-B2}, two other enzymes involved in the metabolism of xenobiotics, may form adducts with benzo(a)pyrene under appropriate conditions. The requirement for reductase and NADPH together with cytochrome P-450 showed that metabolic activation of benzo(a)pyrene preceded binding. This was further suggested by the low level of adduct formation observed in the presence of the cytochrome P-450_{BNF-B2} inhibitors, or when NADPH was replaced by NADH. It was also evident that cytochrome P-450_{PB-B2} did not catalyze the formation of benzo(a)pyrene metabolites that could react with this cytochrome, since there was no binding in the absence of cytochrome P-450_{BNF-B2}. However, it should be pointed out that the cytochrome P-450_{PB-B2} preparation used is quite active in the metabolism of benzo(a)pyrene, producing 7 nmoles of metabolites per min and nmol as determined by HPLC (37).

A previous study by Gozukara et al. (38) has shown that benzo(a)pyrene-7,8-dihydrodiol will form adducts with proteins of the mixed-function oxidase system after metabolic activation. In this case rabbit liver phenobarbital-induced cytochrome P-450 catalyzed the reaction. Covalent binding occurred to this protein and to all other proteins included in the reaction mixtures, i.e. NADPH-cyto-

chrome P-450 reductase, epoxide hydrolase and bovine serum albumin. Thus the target specificity of the dihydrodiol was less than that of activated benzo(a)pyrene in this study. One possible reason for this is that the dihydrodiol is metabolized to the highly reactive 9,10-oxide which will react readily with various macromolecules (1). We have found earlier that after metabolic activation the dihydrodiol will bind more efficiently and less specifically to rat liver microsomal proteins than do several other metabolites of benzo(a)-pyrene (39).

So far we have not determined the ultimate metabolite(s) of benzo(a)pyrene that become bound to the mixed-function oxidase proteins. The 7,8-dihydrodiol-9,10-epoxide is a strong candidate, since it is a major reaction product of cytochrome P-450_{B_{NF}-B₂} (40), but not of cytochrome P-450_{PB-B₂}, and since it will readily form adducts with macromolecules. The previously suggested benzo(a)pyrene-4,5-oxide (41) is less likely to be a major binding species here, since it is formed preferentially by cytochrome P-450_{PB-B₂}, and no binding was observed with this cytochrome. It is possible, however, that several benzo(a)pyrene metabolites bind covalently to proteins in reconstituted systems, although with different efficiencies, as suggested by the binding studies of isolated benzo(a)-pyrene metabolites to microsomes (39).

Apart from benzo(a)pyrene, other substances have also been shown to bind covalently to isolated cytochrome P-450. Both parathion (24) and chloramphenicol (23) formed adducts with cytochrome P-450_{PB-B₂} after metabolic activation by a reconstituted mixed-function oxidase system. Activated chloramphenicol modified amino acid residues and parathion primarily the heme moiety. Both these modifications led to an inactivation of the cytochrome. Whether covalently bound benzo(a)pyrene would have a similar effect on the activity of cytochrome P-450_{B_{NF}-B₂} is not known presently, but the linear time course of covalent binding (cf Fig. 1) might argue against such a possibility.

The covalent binding of benzo(a)pyrene to cytochrome P-450 preferentially occurred to the protein moiety as judged by the electrophoretic behaviour of the radiolabel. The amino acid residues involved in the binding are not known. They are likely to be nucleophiles, however, since reactive metabolites are almost always electrophiles. Thus, lysine appeared to be involved in the binding of chloramphenicol (23,24), as well as in the binding of activated benzo(a)pyrene to histone H1 (42). In addition, methionine is a target for metabolites of acetylaminofluorene and azo dyes (43). Also cysteine and histidine are likely to be targets for electrophilic attack.

Earlier studies have shown that there is little metabolism of benzphetamine in reconstituted mixed-function oxidase system in the absence of phospholipids

(34). The covalent binding of benzo(a)pyrene was inhibited, however, by added phosphatidylcholine. This might be due to a different effect of phospholipids on the metabolism of the more hydrophobic substrate benzo(a)pyrene. It seems less likely that enough phospholipids were present in the enzyme preparations to obtain a maximum metabolism of benzo(a)pyrene if the same amount of phospholipids were required as for the metabolism of benzphetamine (34).

The maximum rate of benzo(a)pyrene binding in the reconstituted system was approximately 0.1 nmol per min and nmol cytochrome P-450_{BNF-B2}. This is slightly lower than the rate observed with liver microsomes from 3-methylcholanthrene-treated rats (28). The amount of benzo(a)pyrene bound on prolonged incubations was more than 6 molecules per molecule cytochrome P-450_{BNF-B2}. The effect of this very high degree of substitution on the properties of the cytochrome is presently being investigated.

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TABLE 1: EFFECT OF COFACTORS AND INHIBITORS ON THE COVALENT BINDING OF BENZO(a)PYRENE TO PROTEINS IN A RECONSTITUTED MIXED-FUNCTION OXIDASE SYSTEM:

Incubations were performed in the presence of 0.05 nmol cytochrome P-450_{BNF-B2}, 0.13 U NADPH-cytochrome P-450 reductase and 10 μ g phosphatidylcholine as described in Methods.

Additions	Concentrations	Benzo(a)pyrene bound (pmol)
Control		161
NADH (-NADPH)	2 mM	13
α -Naphthoflavone	0.1 mM	10
Metirapone	0.1 mM	47

LEGENDS TO FIGURES

Fig. 1. Time course of the covalent binding of benzo(a)pyrene to isolated proteins in reconstituted systems. Cytochrome P-450_{PB-B2} (Δ) or P-450 _{β NF-B2} ($\bullet$) (0.05 nmol) was incubated with 0.13 U NADPH-cytochrome P-450 reductase and 10 μ g phosphatidylcholine as described in Methods.

Fig. 2. Effect of NADPH-cytochrome P-450 reductase on the covalent binding of benzo(a)pyrene to proteins. The incubation mixtures contained 0.05 nmol cytochrome P-450 _{β NF-B2}, 10 μ g phosphatidylcholine and various amounts of reductase.

Fig. 3. Effect of phosphatidylcholine on the covalent binding of benzo(a)pyrene to proteins. The incubation mixtures contained 0.05 nmol cytochrome P-450 _{β NF-B2}, 0.13 U NADPH-cytochrome P-450 reductase and various amounts of phosphatidylcholine.

Fig. 4. Effect of the amount of cytochrome P-450 on the covalent binding of benzo(a)pyrene to proteins in reconstituted systems. The incubation mixtures contained NADPH-cytochrome P-450 reductase in a molar excess of 8 to the cytochrome, 10 μ g phosphatidylcholine, and the amount of cytochrome P-450_{PB-B2} (Δ) or P-450 _{β NF-B2} ($\bullet$) indicated.

Fig. 5. Protein patterns and autoradiographs after SDS-polyacrylamide gel electrophoresis. Lane a, NADPH-cytochrome P-450 reductase; b, cytochrome P-450_{PB-B2}; c, cytochrome P-450 _{β NF-B2}; d, reductase and cytochrome P-450_{PB-B2}; e, reductase and cytochrome P-450 _{β NF-B2}; f, reductase, cytochrome P-450_{PB-B2} and P-450 _{β NF-B2}; g, reductase, cytochrome P-450 _{β NF-B2} and epoxide hydrolase; h, epoxide hydrolase; i, control liver microsomes; j, microsomes from phenobarbital-treated rats; k, microsomes from β -naphthoflavone-treated rats.

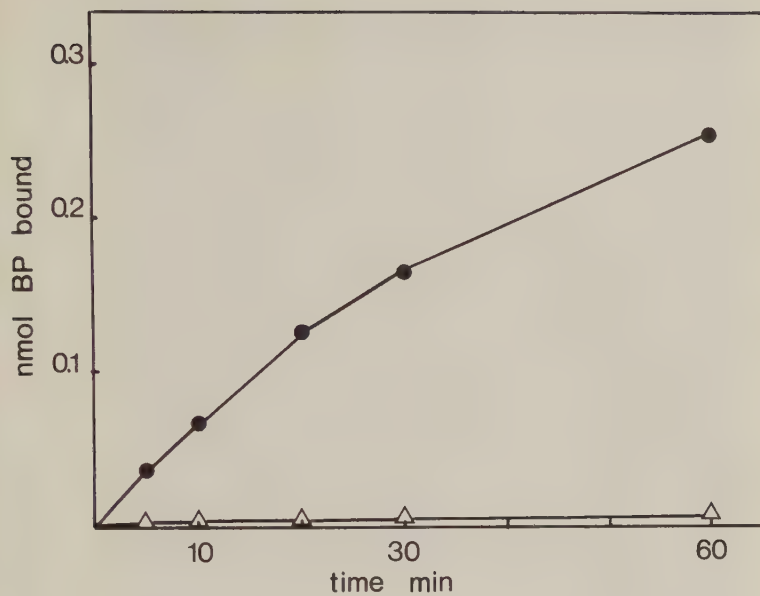
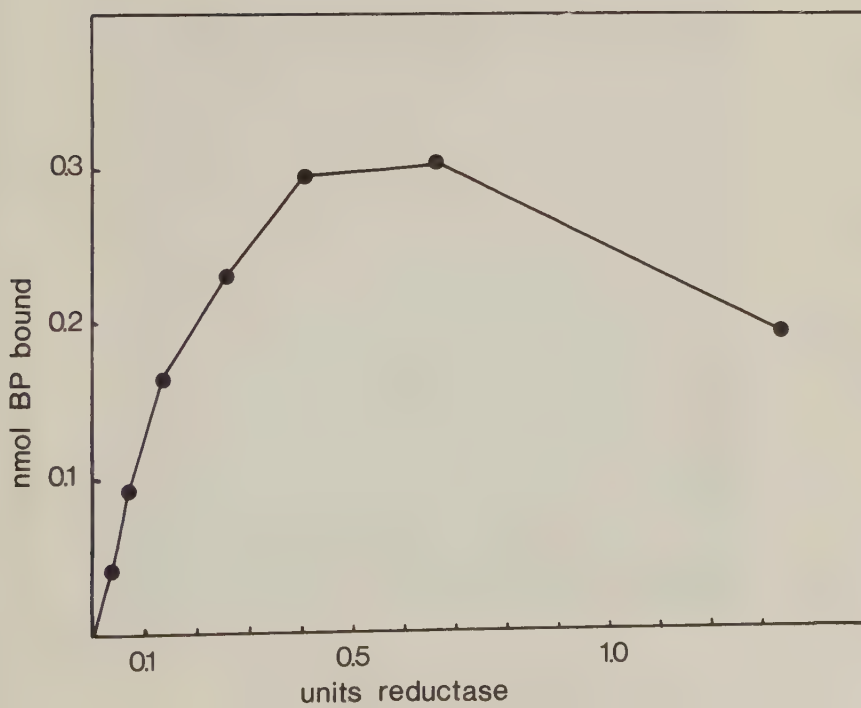
Fig. 1Fig. 2

Fig. 3

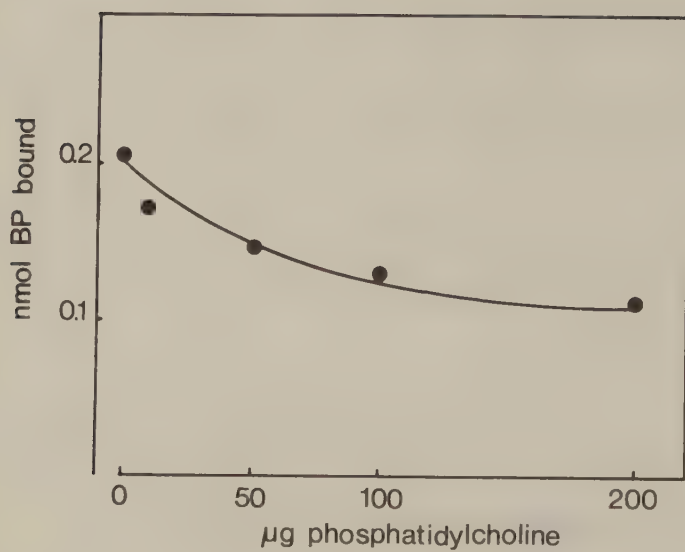
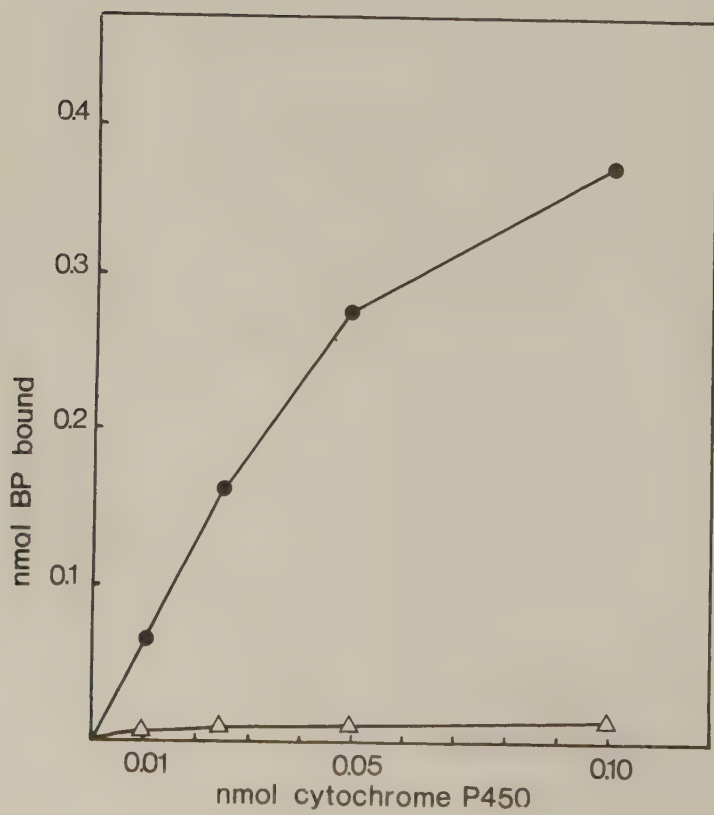
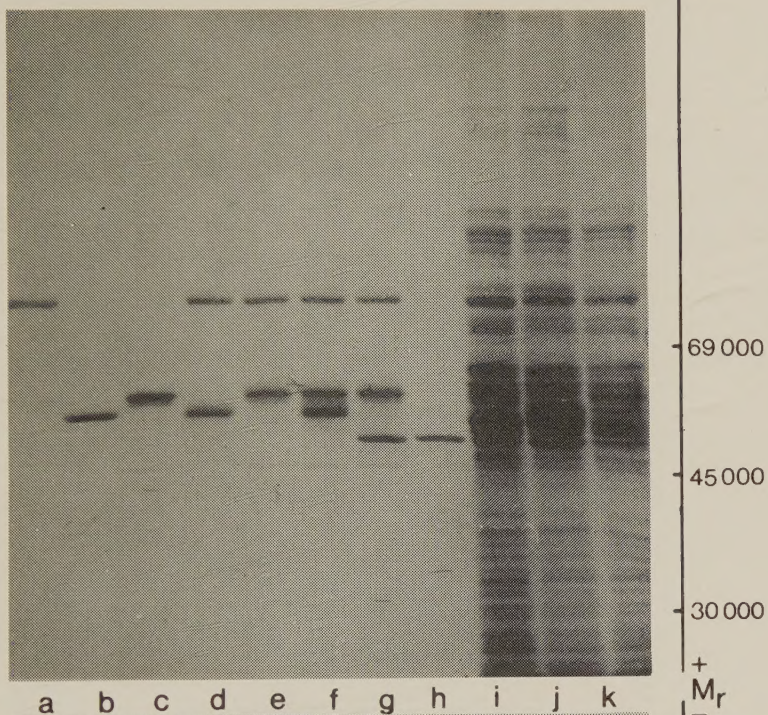


Fig. 4



Protein pattern



Autoradiogram

